INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7:

C12N 15/12, C07K 14/51, 14/495, C12N 15/63, 5/10, C07K 16/22, C12Q 1/68, C12N 15/62, A61K 38/18, A61P 19/10, G01N 33/53, A01K 67/027

(11) International Publication Number:

WO 00/32773

(43) International Publication Date:

8 June 2000 (08.06.00)

(21) International Application Number:

PCT/US99/27990

A1

(22) International Filing Date:

24 November 1999 (24.11.99)

(30) Priority Data:

60/110,283

27 November 1998 (27.11.98) US

(71) Applicant (for all designated States except US): DARWIN DISCOVERY LTD. [GB/GB]; Cambridge Science Park, Milton Road, Cambridge, Cambridgeshire CB4 4WE (GB).

(72) Inventors; and

(75) Inventors/Applicants (for US only): BRUNKOW, Mary, E. [US/US]; 9829 Triton Drive NW, Seattle, WA 98117 (US). GALAS, David, J. [US/US]; 854 Guanajuato Drive, Claremont, CA 91711 (US). KOVACEVICH, Brian [US/US]; 4308 N.E. 6th Place, Renton, WA 98059 (US). MULLI-GAN, John, T. [US/US]; 5823 17th Avenue Northeast, Seattle, WA 98105 (US). PAEPER, Bryan, W. [US/US]: 1617 Summit Avenue #43, Seattle, WA 98122 (US). VAN NESS, Jeffrey [US/US]: 10020 49th Avenue Northeast, Seattle, WA 98125 (US). WINKLER, David, G. [US/US]; 7037 20th Avenue NE, Seattle, WA 98115 (US).

(74) Agent: MCMASTERS, David, D.; Seed and Berry LLP, Suite 6300, 701 Fifth Avenue, Seattle, WA 98104-7092 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: COMPOSITIONS AND METHODS FOR INCREASING BONE MINERALIZATION

(57) Abstract

A novel class or family of $TGF-\beta$ binding proteins is disclosed. Also disclosed are assays for selecting molecules for increasing bone mineralization and methods for utilizing such molecules.

Common Cysteine Backbone

-					
human gremlin.pro				50	
human_cerberus.pro	WIII I I DO				~~~~~~
human_dan.pro	MALLELPOLLV	LLPLOKTTRH	ODGRONOSSL	SPVLLPRNOR	ELPTONHEEA
human_beer.pro					
	52				
human_gramlin.pro		H	CUTAVEMA	111100110	100
human_cerberus.pro	BEKPDLPVAV	PHLVAT. SPA	GEGUDUARKA	LCDDG TDCPA	AEGKKK08Q0
human_dan.pro					
human_beer.pro				WO! D	
_				MODELLA	PCTACTTANA
	101				
human_gremlin.pro	AI.PPPDKAQ	HNDSEQTQ8P	COPGSRNRGR	GOGROTTAMEG	150
human_cerberus.pro	PRZELLBEGI	QSLIQPID.G	MKMBKSPLRP	PAKKPUUUPM	WONTON COM!
human_dan.pro				MLRVLVGAVI	PAMI.T.AADDD
human_beer.pro	AFRVVEGQGW	QAFKNDATEI	I PELGEYPEP	PPELENNKTH	MRACHOODED
human_gremlin.pro	151	V	V	Ψ	¥ 200
human_cerberus.pro	LHVTERKYLK	RDWCKTQPLK	OTIHEEGCNS	RTIINRF.CY	GOCNSPYIPE
human_dan.pro	TOLIVOURAN	WETCKTUPPS	OTITHEGCER	VVVONNI. CO	CVCCCCCCC
human_beer.pro	THYPWELLIN	SAWCEARNIT	OIVGHROCEA	KETOMBA CT	COORDINATION
	HAPPETKOVE	BYSCRELHPT	RYVTDGPCRS	AKPVTELVCS	GOCGPARLLP
	201	J.		AL.	
human_gremlin.pro		cccs are		¥	250
human_cerberus.pro	GAACHERT	SCSPCKP	KKITTMMVTL	NCPELQPPTK	K.KRVTRVKQ
human_dan.pro	TEDOCTECT	SCSHCLP	ARPTINHEPL	NCTELSEVIK	VVMLVEE
human_beer.pro	NATODOLEGE	HCDSCMP	AQSMWEIVTL	ecpaheevpr	VDKLVEKILH
	·	PEGPOPACIP	DRYRAGRYQL	LCPGGBAPRA	rkvrlvas
	that				
human_gremlin.pro	CRC.ISIDLD				300
human_cerberuo.pro	COCKVETENE	DOMITTAGEO	DEPTROUGH		
human_dan.pro	CSCOACOKEP	DGHILHAGSO	CEDCOCCCC		
human_beer.pro	CKCKRLTRFH	SHEGLSVYVQ NQSELKDFOT	CADOPGSQPG	тириририри	PGGGTPEPED
			ANUALYMAN	PRPRARSAKA	nqaelenay.
	301	314			
human_gremlin.pro					
human_cerberus.pro					
human_dan.pro	PPGAPHTEEE	GAED			
human_beer.pro					

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain		or pampinets publishing	internationa	al applications under the
AM AT AU AZ BA BB BE BF BG CA CF CG CH CN CU CZ DE DK EE	Amenia Austria Austria Austriaia Azerbaijan Bosnia and Herzegovina Barbados Belgium Burkina Faso Bulgaria Benin Brazil Belarus Canada Central African Republic Congo Switzerland Côte d'Ivoire Cameroon China Cuba Czech Republic Germany Denmark Estonia	FI FR GA GB GC GR HU IE IL IS IT JP KC KP KZ LC LI LK LR	Spain Finland France Gabon United Kingdom Georgia Ghana Guinea Greece Hungary Ireland Israel Iceland Italy Japan Kenya Kyrgyzstan Democratic People's Republic of Korea Republic of Korea Kazakstan Saint Lucia Liechtenstein Sri Lanka Liberia	LS LT LU LV MC MD MG MK MI MN MR MW MX NE NL NO NZ PL PT RO RU SD SE SG	Lesotho Lithuania Luxembourg Latvia Monaco Republic of Moldova Madagascar The former Yugoslav Republic of Macedonia Mali Mongolia Mauritania Malawi Mexico Niger Netherlands Norway New Zealand Polend Portugal Romania Russian Federation Sudan Sweden Singapore	SI SK SN SZ TD TG TJ TM TR TT UA UG US UZ VN YU ZW	Slovenia Slovakia Senegal Swaziland Chad Togo Tajikistan Turkmenistan Turkey Trinidad and Tobago Ukraine Uganda United States of America Uzbekistan Viet Nam Yugoslavia Zimbabwe

WO 00/32773 PCT/US99/27990

COMPOSITIONS AND METHODS FOR INCREASING BONE MINERALIZATION

TECHNICAL FIELD

10

20

25

The present invention relates generally to pharmaceutical products and methods and, more specifically, to methods and compositions suitable for increasing the mineral content of bone. Such compositions and methods may be utilized to treat a wide variety of conditions, including for example, osteopenia, osteoporosis, fractures and other disorders in which low bone mineral density are a hallmark of the disease.

BACKGROUND OF THE INVENTION

Two or three distinct phases of changes to bone mass occur over the life of an individual (see Riggs, West J. Med. 154:63-77, 1991). The first phase occurs in both men and women, and proceeds to attainment of a peak bone mass. This first phase is achieved through linear growth of the endochondral growth plates, and radial growth due to a rate of periosteal apposition. The second phase begins around age 30 for trabecular bone (flat bones such as the vertebrae and pelvis) and about age 40 for cortical bone (e.g., long bones found in the limbs) and continues to old age. This phase is characterized by slow bone loss, and occurs in both men and women. In women, a third phase of bone loss also occurs, most likely due to postmenopausal estrogen deficiencies. During this phase alone, women may lose an additional 10% of bone mass from the cortical bone and 25% from the trabecular compartment (see Riggs, supra).

Loss of bone mineral content can be caused by a wide variety of conditions, and may result in significant medical problems. For example, osteoporosis is a debilitating disease in humans characterized by marked decreases in skeletal bone mass and mineral density, structural deterioration of bone including degradation of bone microarchitecture and corresponding increases in bone fragility and susceptibility to fracture in afflicted individuals. Osteoporosis in humans is preceded by clinical osteopenia (bone mineral density that is greater than one standard deviation but less than 2.5 standard deviations below the mean value for young adult bone), a condition found in approximately 25 million people in the United States. Another 7-8 million patients in the United States have been diagnosed with clinical osteoporosis (defined as bone mineral content greater than 2.5 standard deviations below that of mature young adult bone). Osteoporosis is one of the most expensive diseases for the health care

15

20

25

30

35

system, costing tens of billions of dollars annually in the United States. In addition to health care-related costs, long-term residential care and lost working days add to the financial and social costs of this disease. Worldwide approximately 75 million people are at risk for osteoporosis.

The frequency of osteoporosis in the human population increases with age, and among Caucasians is predominant in women (who comprise 80% of the osteoporosis patient pool in the United States). The increased fragility and susceptibility to fracture of skeletal bone in the aged is aggravated by the greater risk of accidental falls in this population. More than 1.5 million osteoporosis-related bone fractures are reported in the United States each year. Fractured hips, wrists, and vertebrae are among the most common injuries associated with osteoporosis. Hip fractures in particular are extremely uncomfortable and expensive for the patient, and for women correlate with high rates of mortality and morbidity.

Although osteoporosis has been defined as an increase in the risk of fracture due to decreased bone mass, none of the presently available treatments for skeletal disorders can substantially increase the bone density of adults. There is a strong perception among all physicians that drugs are needed which could increase bone density in adults, particularly in the bones of the wrist, spinal column and hip that are at risk in osteopenia and osteoporosis.

Current strategies for the prevention of osteoporosis may offer some benefit to individuals but cannot ensure resolution of the disease. These strategies include moderating physical activity (particularly in weight-bearing activities) with the onset of advanced age, including adequate calcium in the diet, and avoiding consumption of products containing alcohol or tobacco. For patients presenting with clinical osteopenia or osteoporosis, all current therapeutic drugs and strategies are directed to reducing further loss of bone mass by inhibiting the process of bone absorption, a natural component of the bone remodeling process that occurs constitutively.

For example, estrogen is now being prescribed to retard bone loss. There is, however, some controversy over whether there is any long term benefit to patients and whether there is any effect at all on patients over 75 years old. Moreover, use of estrogen is believed to increase the risk of breast and endometrial cancer.

High doses of dietary calcium, with or without vitamin D has also been suggested for postmenopausal women. However, high doses of calcium can often have unpleasant gastrointestinal side effects, and serum and urinary calcium levels must be continuously monitored (see Khosla and Rigss, Mayo Clin. Proc. 70:978-982, 1995).

Other therapeutics which have been suggested include calcitonin, bisphosphonates, anabolic steroids and sodium fluoride. Such therapeutics however, have undesirable side effects (e.g., calcitonin and steroids may cause nausea and provoke an immune reaction, bisphosphonates and sodium fluoride may inhibit repair of fractures, even though bone density increases modestly) that may prevent their usage (see Khosla and Rigss, supra).

No currently practiced therapeutic strategy involves a drug that stimulates or enhances the growth of new bone mass. The present invention provides compositions and methods which can be utilized to increase bone mineralization, and thus may be utilized to treat a wide variety of conditions where it is desired to increase bone mass. Further, the present invention provides other, related advantages.

SUMMARY OF THE INVENTION

As noted above, the present invention provides a novel class or family of TGF-beta binding-proteins, as well as assays for selecting compounds which increase bone mineral content and bone mineral density, compounds which increase bone mineral content and bone mineral density and methods for utilizing such compounds in the treatment or prevention of a wide variety of conditions.

Within one aspect of the present invention, isolated nucleic acid molecules are provided, wherein said nucleic acid molecules are selected from the group consisting of: (a) an isolated nucleic acid molecule comprising sequence ID Nos. 20 1, 5, 7, 9, 11, 13, or, 15, or complementary sequence thereof; (b) an isolated nucleic acid molecule that specifically hybridizes to the nucleic acid molecule of (a) under conditions of high stringency; and (c) an isolated nucleic acid that encodes a TGF-beta binding-protein according to (a) or (b). Within related aspects of the present invention, isolated nucleic acid molecules are provided based upon hybridization to only a portion 25 of one of the above-identified sequences (e.g., for (a) hybridization may be to a probe of at least 20, 25, 50, or 100 nucleotides selected from nucleotides 156 to 539 or 555 to 687 of Sequence ID No. 1). As should be readily evident, the necessary stringency to be utilized for hybridization may vary based upon the size of the probe. For example, for a 25-mer probe high stringency conditions could include: 60 mM Tris pH 8.0, 2 30 mM EDTA, 5x Denhardt's, 6x SSC, 0.1% (w/v) N-laurylsarcosine, 0.5% (w/v) NP-40 (nonidet P-40) overnight at 45 degrees C, followed by two washes with with 0.2x SSC / 0.1% SDS at 45-50 degrees. For a 100-mer probe under low stringency conditions, suitable conditions might include the following: 5x SSPE, 5x Denhardt's, and 0.5% SDS overnight at 42-50 degrees, followed by two washes with 2x SSPE (or 2x SSC) 35

/0.1% SDS at 42-50 degrees.

10

20

25

30

Within related aspects of the present invention, isolated nucleic acid molecules are provided which have homology to Sequence ID Nos. 1, 5, 7, 9, 11, 13, or 15, at a 50%, 60%, 75%, 80%, 90%, 95%, or 98% level of homology utilizing a Wilbur-Lipman algorithm. Representative examples of such isolated molecules include, for example, nucleic acid molecules which encode a protein comprising Sequence ID NOs. 2, 6, 10, 12, 14, or 16, or have homology to these sequences at a level of 50%, 60%, 75%, 80%, 90%, 95%, or 98% level of homology utilizing a Lipman-Pearson algorithm.

Isolated nucleic acid molecules are typically less than 100kb in size, and, within certain embodiments, less than 50kb, 25kb, 10kb, or even 5kb in size. Further, isolated nucleic acid molecules, within other embodiments, do not exist in a "library" of other unrelated nucleic acid molecules (e.g., a subclone BAC such as described in GenBank Accession No. AC003098 and EMB No. AQ171546). However, isolated nucleic acid molecules can be found in libraries of related molecules (e.g., for shuffling, such as is described in U.S. Patent Nos. 5,837,458; 5,830,721; and 5,811,238). Finally, isolated nucleic acid molecules as described herein do not include nucleic acid molecules which encode Dan, Cerberus, Gremlin, or SCGF (U.S. Patent No. 5,780,263).

Also provided by the present invention are cloning vectors which contain the above-noted nucleic acid molecules, and expression vectors which comprise a promoter (e.g., a regulatory sequence) operably linked to one of the above-noted nucleic acid molecules. Representative examples of suitable promoters include tissue-specific promoters, and viral – based promoters (e.g., CMV-based promoters such as CMV I-E, SV40 early promoter, and MuLV LTR). Expression vectors may also be based upon, or derived from viruses (e.g., a "viral vector"). Representative examples of viral vectors include herpes simplex viral vectors, adenoviral vectors, adenovirus-associated viral vectors and retroviral vectors. Also provided are host cells containing or comprising any of above-noted vectors (including for example, host cells of human, monkey, dog, rat, or mouse origin).

Within other aspects of the present invention, methods of producing TGF-beta binding-proteins are provided, comprising the step of culturing the aforementioned host cell containing vector under conditions and for a time sufficient to produce the TGF-beta binding protein. Within further embodiments, the protein produced by this method may be further purified (e.g., by column chromatography, affinity purification, and the like). Hence, isolated proteins which are encoded by the

15

20

25

30

35

above-noted nucleic acid molecules (e.g., Sequence ID NOs. 2, 4, 6, 8, 10, 12, 14, or 16) may be readily produced given the disclosure of the subject application.

It should also be noted that the aforementioned proteins, or fragments thereof, may be produced as fusion proteins. For example, within one aspect fusion proteins are provided comprising a first polypeptide segment comprising a TGF-beta binding-protein encoded by a nucleic acid molecule as described above, or a portion thereof of at least 10, 20, 30, 50, or 100 amino acids in length, and a second polypeptide segment comprising a non-TGF-beta binding-protein. Within certain embodiments, the second polypeptide may be a tag suitable for purification or recognition (e.g., a polypeptide comprising multiple anionic amino acid residues – see U.S. Patent No. 4,851,341), a marker (e.g., green fluorescent protein, or alkaline phosphatase), or a toxic molecule (e.g., ricin).

Within another aspect of the present invention, antibodies are provided which are capable of specifically binding the above-described class of TGF-beta binding proteins (e.g., human BEER). Within various embodiments, the antibody may be a polyclonal antibody, or a monoclonal antibody (e.g., of human or murine origin). Within further embodiments, the antibody is a fragment of an antibody which retains the binding characteristics of a whole antibody (e.g., an F(ab')₂, F(ab)₂, Fab', Fab, or Fv fragment, or even a CDR). Also provided are hybridomas and other cells which are capable of producing or expressing the aforementioned antibodies.

Within related aspects of the invention, methods are provided detecting a TGF-beta binding protein, comprising the steps of incubating an antibody as described above under conditions and for a time sufficient to permit said antibody to bind to a TGF-beta binding protein, and detecting the binding. Within various embodiments the antibody may be bound to a solid support to facilitate washing or separation, and/or labeled. (e.g., with a marker selected from the group consisting of enzymes, fluorescent proteins, and radioisotopes).

Within other aspects of the present invention, isolated oligonucleotides are provided which hybridize to a nucleic acid molecule according to Sequence ID NOs. 1, 3, 5, 7, 9, 11, 13, 15, 17, or 18 or the complement thereto, under conditions of high stringency. Within further embodiments, the oligonucleotide may be found in the sequence which encodes Sequence ID Nos. 2, 4, 6, 8, 10, 12, 14, or 16. Within certain embodiments, the oligonucleotide is at least 15, 20, 30, 50, or 100 nucleotides in length. Within further embodiments, the oligonucleotide is labeled with another molecule (e.g., an enzyme, fluorescent molecule, or radioisotope). Also provided are primers which are capable of specifically amplifying all or a portion of the above-

15

20

25

30

35

mentioned nucleic acid molecules which encode TGF-beta binding-proteins. As utilized herein, the term "specifically amplifying" should be understood to refer to primers which amplify the aforementioned TGF-beta binding-proteins, and not other TGF-beta binding proteins such as Dan, Cerberus, Gremlin, or SCGF (U.S. Patent No. 5,780,263).

Within related aspects of the present invention, methods are provided for detecting a nucleic acid molecule which encodes a TGF-beta binding protein, comprising the steps of incubating an oligonucleotide as described above under conditions of high stringency, and detecting hybridization of said oligonucleotide. Within certain embodiments, the oligonucleotide may be labeled and/or bound to a solid support.

Within other aspects of the present invention, ribozymes are provided which are capable of cleaving RNA which encodes one of the above-mentioned TGF-beta binding-proteins (e.g., Sequence ID NOs. 2, 6, 8, 10, 12, 14, or 16). Such ribozymes may be composed of DNA, RNA (including 2'-O-methyl ribonucleic acids), nucleic acid analogs (e.g., nucleic acids having phosphorothioate linkages) or mixtures thereof. Also provided are nucleic acid molecules (e.g., DNA or cDNA) which encode these ribozymes, and vectors which are capable of expressing or producing the ribozymes. Representative examples of vectors include plasmids, retrotransposons, cosmids, and viral-based vectors (e.g., viral vectors generated at least in part from a retrovirus, adenovirus, or, adeno-associated virus). Also provided are host cells (e.g., human, dog, rat, or mouse cells) which contain these vectors. In certain embodiments, the host cell may be stably transformed with the vector.

Within further aspects of the invention, methods are provided for producing ribozymes either synthetically, or by in vitro or in vivo transcription. Within further embodiments, the ribozymes so produced may be further purified and/or formulated into pharmaceutical compositions (e.g., the ribozyme or nucleic acid molecule encoding the ribozyme along with a pharmaceutically acceptable carrier or diluent). Similarly, the antisense oligonucleotides and antibodies or other selected molecules described herein may be formulated into pharmaceutical compositions.

Within other aspects of the present invention, antisense oligonucleotides are provided comprising a nucleic acid molecule which hybridizes to a nucleic acid molecule according to Sequence ID NOs. 1, 3, 5, 7, 9, 11, 13, or 15, or the complement thereto, and wherein said oligonucleotide inhibits the expression of TGF-beta binding protein as described herein (e.g., human BEER). Within various embodiments, the oligonucleotide is 15, 20, 25, 30, 35, 40, or 50 nucleotides in length. Preferably, the

15

20

30

oligonucleotide is less than 100, 75, or 60 nucleotides in length. As should be readily evident, the oligonucleotide may be comprised of one or more nucleic acid analogs, ribonucleic acids, or deoxyribonucleic acids. Further, the oligonucleotide may be modified by one or more linkages, including for example, covalent linkage such as a phosphorothioate linkage, a phosphotriester linkage, a methyl phosphonate linkage, a methylene(methylimino) linkage, a morpholino linkage, an amide linkage, a polyamide linkage, a short chain alkyl intersugar linkage, a cycloalkyl intersugar linkage, a short chain heteroatomic intersugar linkage and a heterocyclic intersugar linkage. One representative example of a chimeric oligonucleotide is provied in U.S. Patent No. 5,989,912.

Within yet another aspect of the present invention, methods are provided for increasing bone mineralization, comprising introducing into a warm-blooded animal an effective amount of the ribozyme as described above. Within related aspects, such methods comprise the step of introducing into a patient an effective amount of the nucleic acid molecule or vector as described herein which is capable of producing the desired ribozyme, under conditions favoring transcription of the nucleic acid molecule to produce the ribozyme.

Within other aspects of the invention transgenic, non-human animals are provided. Within one embodiment a transgenic animal is provided whose germ cells and somatic cells contain a nucleic acid molecule encoding a TGF-beta binding-protein as described above which is operably linked to a promoter effective for the expression of the gene, the gene being introduced into the animal, or an ancestor of the animal, at an embryonic stage, with the proviso that said animal is not a human. Within other embodiments, transgenic knockout animals are provided, comprising an animal whose germ cells and somatic cells comprise a disruption of at least one allele of an endogenous nucleic acid molecule which hybridizes to a nucleic acid molecule which encodes a TGF-binding protein as described herein, wherein the disruption prevents transcription of messenger RNA from said allele as compared to an animal without the disruption, with the proviso that the animal is not a human. Within various embodiments, the disruption is a nucleic acid deletion, substitution, or, insertion. Within other embodiments the transgenic animal is a mouse, rat, sheep, pig, or dog.

Within further aspects of the invention, kits are provided for the detection of TGF-beta binding-protein gene expression, comprising a container that comprises a nucleic acid molecule, wherein the nucleic acid molecule is selected from the group consisting of (a) a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, or 15; (b) a nucleic acid molecule comprising the

complement of the nucleotide sequence of (a); (c) a nucleic acid molecule that is a fragment of (a) or (b) of at least 15, 20 30, 50, 75, or, 100 nucleotides in length. Also provided are kits for the detection of a TGF-beta binding-protein which comprise a container that comprise one of the TGF-beta binding protein antibodies described herein.

For example, within one aspect of the present invention methods are provided for determining whether a selected molecule is capable of increasing bone mineral content, comprising the steps of (a) mixing one or more candidate molecules with TGF-beta-binding-protein encoded by the nucleic acid molecule according to claim 1 and a selected member of the TGF-beta family of proteins (e.g., BMP 5 or 6), (b) determining whether the candidate molecule alters the signaling of the TGF-beta family member, or alters the binding of the TGF-beta binding-protein to the TGF-beta family member. Within certain embodiments, the molecule alters the ability of TGF-beta to function as a positive regulator of mesenchymal cell differentiation. Within this aspect of the present invention, the candidate molecule(s) may alter signaling or binding by, for example, either decreasing (e.g., inhibiting), or increasing (e.g., enhancing) signaling or binding.

Within yet another aspect, methods are provided for determining whether a selected molecule is capable of increasing bone mineral content, comprising the step of determining whether a selected molecule inhibits the binding of TGF-beta binding-protein to bone, or an analogue thereof. Representative examples of bone or analogues thereof include hydroxyapatite and primary human bone samples obtained via biopsy.

Within certain embodiments of the above-recited methods, the selected molecule is contained within a mixture of molecules and the methods may further comprise the step of isolating one or more molecules which are functional within the assay. Within yet other embodiments, TGF-beta family of proteins is bound to a solid support and the binding of TGF-beta binding-protein is measured or TGF-beta binding-protein are bound to a solid support and the binding of TGF-beta proteins are measured.

Utilizing methods such as those described above, a wide variety of molecules may be assayed for their ability to increase bone mineral content by inhibiting the binding of the TGF-beta binding-protein to the TGF-beta family of proteins. Representative examples of such molecules include proteins or peptides, organic molecules, and nucleic acid molecules.

Within other related aspects of the invention, methods are provided for increasing bone mineral content in a warm-blooded animal, comprising the step of

25

30

20

25

administering to a warm-blooded animal a therapeutically effective amount of a molecule identified from the assays recited herein. Within another aspect, methods are provided for increasing bone mineral content in a warm-blooded animal, comprising the step of administering to a warm-blooded animal a therapeutically effective amount of a molecule which inhibits the binding of the TGF-beta binding-protein to the TGF-beta super-family of proteins, including bone morphogenic proteins (BMPs). Representative examples of suitable molecules include antisense molecules, ribozymes, ribozyme genes, and antibodies (e.g., a humanized antibody) which specifically recognize and alter the activity of the TGF-beta binding-protein.

Within another aspect of the present invention, methods are provided for increasing bone mineral content in a warm-blooded animal, comprising the steps of (a) introducing into cells which home to the bone a vector which directs the expression of a molecule which inhibits the binding of the TGF-beta binding-protein to the TGF-beta family of proteins and bone morphogenic proteins (BMPs), and (b) administering the vector-containing cells to a warm-blooded animal. As utilized herein, it should be understood that cells "home to bone" if they localize within the bone matrix after peripheral administration. Within one embodiment, such methods further comprise, prior to the step of introducing, isolating cells from the marrow of bone which home to the bone. Within a further embodiment, the cells which home to bone are selected from the group consisting of CD34+ cells and osteoblasts.

Within other aspects of the present invention, molecules are provided (preferably isolated) which inhibit the binding of the TGF-beta binding-protein to the TGF-beta super-family of proteins.

Within further embodiments, the molecules may be provided as a composition, and can further comprise an inhibitor of bone resorption. Representative examples of such inhibitors include calcitonin, estrogen, a bisphosphonate, a growth factor having anti-resorptive activity and tamoxifen.

Representative examples of molecules which may be utilized in the afore-mentioned therapeutic contexts include, e.g., ribozymes, ribozyme genes, antisense molecules, and/or antibodies (e.g., humanized antibodies). Such molecules may depending upon their selection, used to alter, antagonize, or agonize the signalling or binding of a TGF-beta binding-protein family member as described herein

Within various embodiments of the invention, the above-described molecules and methods of treatment or prevention may be utilized on conditions such as osteoporosis, osteomalasia, periodontal disease, scurvy, Cushing's Disease, bone fracture and conditions due to limb immobilization and steroid usage.

15

20

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth herein which describe in more detail certain procedures or compositions (e.g., plasmids, etc.), and are therefore incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic illustration comparing the amino acid sequence of Human Dan; Human Gremlin; Human Cerberus and Human Beer. Arrows indicate the Cysteine backbone.

Figure 2 summarizes the results obtained from surveying a variety of human tissues for the expression of a TGF-beta binding-protein gene, specifically, the Human Beer gene. A semi-quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) procedure was used to amplify a portion of the gene from first-strand cDNA synthesized from total RNA (described in more detail in EXAMPLE 2A).

Figure 3 summarizes the results obtained from RNA in situ hybridization of mouse embryo sections, using a cRNA probe that is complementary to the mouse Beer transcript (described in more detail in EXAMPLE 2B). Panel A is a transverse section of 10.5 dpc embryo. Panel B is a sagittal section of 12.5 dpc embryo and panels C and D are sagittal sections of 15.5 dpc embryos.

Figure 4 illustrates, by western blot analysis, the specificity of three different polyclonal antibodies for their respective antigens (described in more detail in EXAMPLE 4). Figure 4A shows specific reactivity of an anti-H. Beer antibody for H. Beer antibody for H. Dan or H. Gremlin. Figure 4B shows reactivity of an anti-H. Gremlin antibody for H. Gremlin antigen, but not H. Beer or H. Dan. Figure 4C shows reactivity of an anti-H. Dan antibody for H. Dan, but not H. Beer or H. Gremlin.

Figure 5 illustrates, by western blot analysis, the selectivity of the TGF-beta binding-protein, Beer, for BMP-5 and BMP-6, but not BMP-4 (described in more detail in EXAMPLE 5).

Figure 6 demonstrates that the ionic interaction between the TGF-beta binding-protein, Beer, and BMP-5 has a dissociation constant in the 15-30 nM range.

15

20

30

35

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

Prior to setting forth the invention in detail, it may be helpful to an understanding thereof to set forth definitions of certain terms and to list and to define the abbreviations that will be used hereinafter.

"Molecule" should be understood to include proteins or peptides (e.g., antibodies, recombinant binding partners, peptides with a desired binding affinity), nucleic acids (e.g., DNA, RNA, chimeric nucleic acid molecules, and nucleic acid analogues such as PNA); and organic or inorganic compounds.

"TGF-beta" should be understood to include any known or novel member of the TGF-beta super-family, which also includes bone morphogenic proteins (BMPs).

"TGF-beta receptor" should be understood to refer to the receptor specific for a particular member of the TGF-beta super-family (including bone morphogenic proteins (BMPs)).

"TGF-beta binding-protein" should be understood to refer to a protein with specific binding affinity for a particular member or subset of members of the TGF-beta super-family (including bone morphogenic proteins (BMPs)). Specific examples of TGF-beta binding-proteins include proteins encoded by Sequence ID Nos. 1, 5, 7, 9, 11, 13, and 15.

Inhibiting the "binding of the TGF-beta binding-protein to the TGF-beta family of proteins and bone morphogenic proteins (BMPs)" should be understood to refer to molecules which allow the activation of TGF-beta or bone morphogenic proteins (BMPs), or allow the binding of TGF-beta family members including bone morphogenic proteins (BMPs) to their respective receptors, by removing or preventing TGF-beta from binding to TGF-binding-protein. Such inhibition may be accomplished, for example, by molecules which inhibit the binding of the TGF-beta binding-protein to specific members of the TGF-beta super-family.

"Vector" refers to an assembly which is capable of directing the expression of desired protein. The vector must include transcriptional promoter elements which are operably linked to the gene(s) of interest. The vector may be composed of either deoxyribonucleic acids ("DNA"), ribonucleic acids ("RNA"), or a combination of the two (e.g., a DNA-RNA chimeric). Optionally, the vector may include a polyadenylation sequence, one or more restriction sites, as well as one or more selectable markers such as neomycin phosphotransferase or hygromycin phosphotransferase. Additionally, depending on the host cell chosen and the vector

15

20

25

30

employed, other genetic elements such as an origin of replication, additional nucleic acid restriction sites, enhancers, sequences conferring inducibility of transcription, and selectable markers, may also be incorporated into the vectors described herein.

An "isolated nucleic acid molecule" is a nucleic acid molecule that is not integrated in the genomic DNA of an organism. For example, a DNA molecule that encodes a TGF-binding protein that has been separated from the genomic DNA of a eukaryotic cell is an isolated DNA molecule. Another example of an isolated nucleic acid molecule is a chemically-synthesized nucleic acid molecule that is not integrated in the genome of an organism. The isolated nucleic acid molecule may be genomic DNA, cDNA, RNA, or composed at least in part of nucleic acid analogs.

An "isolated polypeptide" is a polypeptide that is essentially free from contaminating cellular components, such as carbohydrate, lipid, or other proteinaceous impurities associated with the polypeptide in nature. Within certain embodiments, a particular protein preparation contains an isolated polypeptide if it appears nominally as a single band on SDS-PAGE gel with Coomassie Blue staining. "Isolated" when referring to organic molecules means that the compounds are greater than 90 percent pure utilizing methods which are well known in the art (e.g., NMR, melting point).

"Sclerosteosis" Sclerosteosis is a term that was applied by Hansen (1967) (Hansen, H. G., Sklerosteose In: Opitz, H.; Schmid, F., Handbuch der Kinderheilkunde. Berlin: Springer (pub.) 6 1967. Pp. 351-355) to a disorder similar to van Buchem hyperostosis corticalis generalisata but possibly differing in radiologic appearance of the bone changes and in the presence of asymmetric cutaneous syndactyly of the index and middle fingers in many cases. The jaw has an unusually square appearance in this condition.

"<u>Humanized antibodies</u>" are recombinant proteins in which murine complementary determining regions of monoclonal antibodies have been transferred from heavy and light variable chains of the murine immunoglobulin into a human variable domain.

As used herein, an "antibody fragment" is a portion of an antibody such as F(ab')₂, F(ab)₂, Fab', Fab, and the like. Regardless of structure, an antibody fragment binds with the same antigen that is recognized by the intact antibody. For example, an anti-TGF-beta binding-protein monoclonal antibody fragment binds with an epitope of TGF-beta binding-protein.

The term "antibody fragment" also includes any synthetic or genetically engineered protein that acts like an antibody by binding to a specific antigen to form a complex. For example, antibody fragments include isolated fragments consisting of the

light chain variable region, "Fv" fragments consisting of the variable regions of the heavy and light chains, recombinant single chain polypeptide molecules in which light and heavy variable regions are connected by a peptide linker ("sFv proteins"), and minimal recognition units consisting of the amino acid residues that mimic the hypervariable region.

A "detectable label" is a molecule or atom which can be conjugated to an antibody moiety to produce a molecule useful for diagnosis. Examples of detectable labels include chelators, photoactive agents, radioisotopes, fluorescent agents, paramagnetic ions, enzymes, and other marker moieties.

As used herein, an "<u>immunoconjugate</u>" is a molecule comprising an anti-TGF-beta binding-protein antibody, or an antibody fragment, and a detectable label. An immunoconjugate has roughly the same, or only slightly reduced, ability to bind TGF-beta binding-protein after conjugation as before conjugation.

Abbreviations: TGF-beta – "Transforming Growth Factor-beta"; TGF-bBP – "Transforming Growth Factor-beta binding-protein" (one representative TGF-bBP is designated "H. Beer"); BMP – "bone morphogenic protein"; PCR – "polymerase chain reaction"; RT-PCR - PCR process in which RNA is first transcribed into DNA at the first step using reverse transcriptase (RT); cDNA - any DNA made by copying an RNA sequence into DNA form.

20

25

30

10

15

As noted above, the present invention provides a novel class of TGF-beta binding-proteins, as well as methods and compositions for increasing bone mineral content in warm-blooded animals. Briefly, the present inventions are based upon the unexpected discovery that a mutation in the gene which encodes a novel member of the TGF-beta binding-protein family results in a rare condition (sclerosteosis) characterized by bone mineral contents which are one- to four-fold higher than in normal individuals. Thus, as discussed in more detail below this discovery has led to the development of assays which may be utilized to select molecules which inhibit the binding of the TGF-beta binding-protein to the TGF-beta family of proteins and bone morphogenic proteins (BMPs), and methods of utilizing such molecules for increasing the bone mineral content of warm-blooded animals (including for example, humans).

DISCUSSION OF THE DISEASE KNOWN AS SCLEROSTEOSIS

Sclerosteosis is a term that was applied by Hansen (1967) (Hansen, H. G., Sklerosteose In: Opitz, H.; Schmid, F., Handbuch der Kinderheilkunde. Berlin: Springer (pub.) 6 1967. Pp. 351-355) to a disorder similar to van Buchem hyperostosis

20

25

corticalis generalisata but possibly differing in radiologic appearance of the bone changes and in the presence of asymmetric cutaneous syndactyly of the index and middle fingers in many cases.

Sclerosteosis is now known to be an autosomal semi-dominant disorder which is characterized by widely disseminated sclerotic lesions of the bone in the adult. The condition is progressive. Sclerosteosis also has a developmental aspect which is associated with syndactyly (two or more fingers are fused together). The Sclerosteosis Syndrome is associated with large stature and many affected individuals attain a height of six feet or more. The bone mineral content of homozygotes can be 1 to 6 fold over normal individuals and bone mineral density can be 1 to 4 fold above normal values (e.g., from unaffected siblings).

The Sclerosteosis Syndrome occurs primarily in Afrikaaners of Dutch descent in South Africa. Approximately 1/140 individuals in the Afrikaaner population are carriers of the mutated gene (heterozygotes). The mutation shows 100% penetrance. There are anecdotal reports of increased of bone mineral density in heterozygotes with no associated pathologies (syndactyly or skull overgrowth).

It appears at the present time that there is no abnormality of the pituitary-hypothalamus axis in Sclerosteosis. In particular, there appears to be no over-production of growth hormone and cortisone. In addition, sex hormone levels are normal in affected individuals. However, bone turnover markers (osteoblast specific alkaline phosphatase, osteocalcin, type 1 procollagen C' propeptide (PICP), and total alkaline phosphatase; (see Comier, C., Curr. Opin. in Rheu. 7:243, 1995) indicate that there is hyperosteoblastic activity associated with the disease but that there is normal to slightly decreased osteoclast activity as measured by markers of bone resorption (pyridinoline, deoxypryridinoline, N-telopeptide, urinary hydroxyproline, plasma tartrate-resistant acid phosphatases and galactosyl hydroxylysine (see Comier, supra)).

Sclerosteosis is characterized by the continual deposition of bone throughout the skeleton during the lifetime of the affected individuals. In homozygotes the continual deposition of bone mineral leads to an overgrowth of bone in areas of the skeleton where there is an absence of mechanoreceptors (skull, jaw, cranium). In homozygotes with Sclerosteosis, the overgrowth of the bones of the skull leads to cranial compression and eventually to death due to excessive hydrostatic pressure on the brain stem. In all other parts of the skeleton there is a generalized and diffuse sclerosis. Cortical areas of the long bones are greatly thickened resulting in a substantial increase in bone strength. Trabecular connections are increased in thickness

which in turn increases the strength of the trabecular bone. Sclerotic bones appear unusually opaque to x-rays.

As described in more detail in Example 1, the rare genetic mutation that is responsible for the Sclerosteosis syndrome has been localized to the region of human chromosome 17 that encodes a novel member of the TGF-beta binding-protein family (one representative example of which is designated "H. Beer"). As described in more detail below, based upon this discovery, the mechanism of bone mineralization is more fully understood, allowing the development of assays for molecules which increase bone mineralization, and use of such molecules to increase bone mineral content, and in the treatment or prevention of a wide number of diseases.

TGF-BETA SUPER-FAMILY

The Transforming Growth Factor-beta (TGF-beta) super-family contains a variety of growth factors that share common sequence elements and structural motifs (at both the secondary and tertiary levels). This protein family is known to exert a wide spectrum of biological responses on a large variety of cell types. Many of them have important functions during the embryonal development in pattern formation and tissue specification; in adults they are involved, e.g., in wound healing and bone repair and bone remodeling, and in the modulation of the immune system. In addition to the three TGF-beta's, the super-family includes the Bone Morphogenic Proteins (BMPs), Activins, Inhibins, Growth and Differentiation Factors (GDFs), and Glial-Derived Neurotrophic Factors (GDNFs). Primary classification is established through general sequence features that bin a specific protein into a general sub-family. Additional stratification within the sub-family is possible due to stricter sequence conservation between members of the smaller group. In certain instances, such as with BMP-5, BMP-6 and BMP-7, this can be as high as 75 percent amino acid homology between 25 members of the smaller group. This level of identity enables a single representative sequence to illustrate the key biochemical elements of the sub-group that separates it from other members of the larger family.

TGF-beta signals by inducing the formation of hetero-oligomeric complexes of type I and type II receptors. The crystal structure of TGF-beta2 has been determined. The general fold of the TGF-beta2 monomer contains a stable, compact, cysteine knotlike structure formed by three disulphide bridges. Dimerization, stabilized by one disulphide bridge, is antiparallel.

TGF-beta family members initiate their cellular action by binding to receptors with intrinsic serine/threonine kinase activity. This receptor family consists

WO 00/32773

10

20

25

30

of two subfamilies, denoted type I and type II receptors. Each member of the TGF-beta family binds to a characteristic combination of type I and type II receptors, both of which are needed for signaling. In the current model for TGF-beta receptor activation, TGF-beta first binds to the type II receptor (TbR-II), which occurs in the cell membrane in an oligomeric form with activated kinase. Thereafter, the type I receptor (TbR-I), which can not bind ligand in the absence of TbR-II, is recruited into the complex. TbR-II then phosphorylates TbR-I predominantly in a domain rich in glycine and serine residues (GS domain) in the juxtamembrane region, and thereby activates TbR-I

Thus far seven type I receptors and five type II receptors have been identified.

BONE MORPHOGENIC PROTEINS (BMPS) ARE KEY REGULATORY PROTEINS IN **DETERMINING BONE MINERAL DENSITY IN HUMANS**

A major advance in the understanding of bone formation was identification of the bone morphogenic proteins (BMPs), also known as osteogenic 15 proteins (OPs), which regulate cartilage and bone differentiation in vivo. BMPs/OPs induce endochondral bone differentiation through a cascade of events which include formation of cartilage, hypertrophy and calcification of the cartilage, vascular invasion, differentiation of osteoblasts, and formation of bone. As described above, the BMPs/OPs (BMP 2-14, and osteogenic protein 1 and -2, OP-1 and OP-2) are members of the TGF-beta super-family. The striking evolutionary conservation between members the BMP/OP sub-family suggests that they are critical in the normal development and function of animals. Moreover, the presence of multiple forms of BMPs/OPs raises an important question about the biological relevance of this apparent redundancy. In addition to postfetal chondrogenesis and osteogenesis, the BMPs/OPs play multiple roles in skeletogenesis (including the development of craniofacial and dental tissues) and in embryonic development and organogenesis of parenchymatous organs, including the kidney. It is now understood that nature relies on common (and few) molecular mechanisms tailored to provide the emergence of specialized tissues and organs. The BMP/OP super-family is an elegant example of nature parsimony in programming multiple specialized functions deploying molecular isoforms with minor variation in amino acid motifs within highly conserved carboxy-terminal regions.

BMP ANTAGONISM

The BMP and Activin sub-families are subject to significant post-

WO 00/32773

translational regulation. An intricate extracellular control system exists, whereby a high affinity antagonist is synthesized and exported, and subsequently complexes selectively with BMPs or activins to disrupt their biological activity (W.C. Smith (1999) TIG 15(1) 3-6). A number of these natural antagonists have been identified, and based on sequence divergence appear to have evolved independently due to the lack of primary sequence conservation. There has been no structural work to date on this class of proteins. Studies of these antagonists has highlighted a distinct preference for interacting and neutralizing BMP-2 and BMP-4. Furthermore, the mechanism of inhibition seems to differ for the different antagonists (S. Iemura et al. (1998) *Proc Natl Acad Sci USA 95* 9337-9342).

NOVEL TGF-BETA BINDING-PROTEINS

1. Background re: TGF-beta binding-proteins

As noted above, the present invention provides a novel class of TGF-beta binding-proteins that possess a nearly identical cysteine (disulfide) scaffold when compared to Human DAN, Human Gremlin, and Human Cerberus, and SCGF (U.S. Patent No. 5,780,263) but almost no homology at the nucleotide level (for background information, see generally Hsu, D.R., Economides, A.N., Wang, X., Eimon, P.M., Harland, R.M., "The *Xenopus* Dorsalizing Factor Gremlin Identifies a Novel Family of Secreted Proteins that Antagonize BMP Activities," *Molecular Cell* 1:673-683, 1998).

One representative example of the novel class of TGF-beta binding-proteins is disclosed in Sequence ID Nos. 1, 5, 9, 11, 13, and 15. Representative members of this class of binding proteins should also be understood to include variants of the TGF-beta binding-protein (e.g., Sequence ID Nos. 5 and 7). As utilized herein, a "TGF-beta binding-protein variant gene" refers to nucleic acid molecules that encode a polypeptide having an amino acid sequence that is a modification of SEQ ID Nos: 2, 10, 12, 14 or 16. Such variants include naturally-occurring polymorphisms or allelic variants of TGF-beta binding-protein genes, as well as synthetic genes that contain conservative amino acid substitutions of these amino acid sequences. Additional variant forms of a TGF-beta binding-protein gene are nucleic acid molecules that contain insertions or deletions of the nucleotide sequences described herein. TGF-beta binding-protein variant genes can be identified by determining whether the genes hybridize with a nucleic acid molecule having the nucleotide sequence of SEQ ID Nos: 1, 5, 7, 9, 11, 13, or 15 under stringent conditions. In addition, TGF-beta binding-protein variant genes should encode a protein having a cysteine backbone.

15

20

25

As an alternative, TGF-beta binding-protein variant genes can be identified by sequence comparison. As used herein, two amino acid sequences have "100% amino acid sequence identity" if the amino acid residues of the two amino acid sequences are the same when aligned for maximal correspondence. Similarly, two nucleotide sequences have "100% nucleotide sequence identity" if the nucleotide residues of the two nucleotide sequences are the same when aligned for maximal correspondence. Sequence comparisons can be performed using standard software programs such as those included in the LASERGENE bioinformatics computing suite, which is produced by DNASTAR (Madison, Wisconsin). Other methods for comparing two nucleotide or amino acid sequences by determining optimal alignment are well-known to those of skill in the art (see, for example, Peruski and Peruski, The Internet and the New Biology: Tools for Genomic and Molecular Research (ASM Press, Inc. 1997), Wu et al. (eds.), "Information Superhighway and Computer Databases of Nucleic Acids and Proteins," in Methods in Gene Biotechnology, pages 123-151 (CRC Press, Inc. 1997), and Bishop (ed.), Guide to Human Genome Computing, 2nd Edition (Academic Press, Inc. 1998)).

A variant TGF-beta binding-protein should have at least a 50% amino acid sequence identity to SEQ ID NOs: 2, 6, 10, 12, 14 or 16 and preferably, greater than 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% identity. Alternatively, TGF-beta binding-protein variants can be identified by having at least a 70% nucleotide sequence identity to SEQ ID NOs: 1, 5, 9, 11, 13 or 15. Moreover, the present invention contemplates TGF-beta binding-protein gene variants having greater than 75%, 80%, 85%, 90%, or 95% identity to SEQ ID NO:1. Regardless of the particular method used to identify a TGF-beta binding-protein variant gene or variant TGF-beta binding-protein, a variant TGF-beta binding-protein or a polypeptide encoded by a variant TGF-beta binding-protein gene can be functionally characterized by, for example, its ability to bind to and/or inhibit the signaling of a selected member of the TGF-beta family of proteins, or by its ability to bind specifically to an anti-TGF-beta binding-protein antibody.

The present invention includes functional fragments of TGF-beta binding-protein genes. Within the context of this invention, a "functional fragment" of a TGF-beta binding-protein gene refers to a nucleic acid molecule that encodes a portion of a TGF-beta binding-protein polypeptide which either (1) possesses the above-noted function activity, or (2) specifically binds with an anti-TGF-beta binding-protein antibody. For example, a functional fragment of a TGF-beta binding-protein gene described herein comprises a portion of the nucleotide sequence of SEQ ID Nos:

20

25

30

10

15

20

25

30

35

1, 5, 9, 11, 13, or 15.

Isolation of the TGF-beta binding-protein gene

DNA molecules encoding a binding-protein gene can be obtained by screening a human cDNA or genomic library using polynucleotide probes based upon, for example, SEQ ID NO:1.

For example, the first step in the preparation of a cDNA library is to isolate RNA using methods well-known to those of skill in the art. In general, RNA isolation techniques must provide a method for breaking cells, a means of inhibiting RNase-directed degradation of RNA, and a method of separating RNA from DNA, protein, and polysaccharide contaminants. For example, total RNA can be isolated by freezing tissue in liquid nitrogen, grinding the frozen tissue with a mortar and pestle to lyse the cells, extracting the ground tissue with a solution of phenol/chloroform to remove proteins, and separating RNA from the remaining impurities by selective precipitation with lithium chloride (see, for example, Ausubel et al. (eds.), Short Protocols in Molecular Biology, 3rd Edition, pages 4-1 to 4-6 (John Wiley & Sons 1995) ["Ausubel (1995)"], Wu et al., Methods in Gene Biotechnology, pages 33-41 (CRC Press, Inc. 1997) ["Wu (1997)"]).

Alternatively, total RNA can be isolated by extracting ground tissue with guanidinium isothiocyanate, extracting with organic solvents, and separating RNA from contaminants using differential centrifugation (see, for example, Ausubel (1995) at pages 4-1 to 4-6; Wu (1997) at pages 33-41).

In order to construct a cDNA library, poly(A) RNA must be isolated from a total RNA preparation. Poly(A) RNA can be isolated from total RNA by using the standard technique of oligo(dT)-cellulose chromatography (see, for example, Ausubel (1995) at pages 4-11 to 4-12).

Double-stranded cDNA molecules are synthesized from poly(A)⁺ RNA using techniques well-known to those in the art. (see, for example, Wu (1997) at pages 41-46). Moreover, commercially available kits can be used to synthesize double-stranded cDNA molecules. For example, such kits are available from Life Technologies, Inc. (Gaithersburg, Maryland), CLONTECH Laboratories, Inc. (Palo Alto, California), Promega Corporation (Madison, Wisconsin) and Stratagene Cloning Systems (La Jolla, California).

The basic approach for obtaining TGF-beta binding-protein cDNA clones can be modified by constructing a subtracted cDNA library which is enriched in TGF-binding-protein-specific cDNA molecules. Techniques for constructing subtracted libraries are well-known to those of skill in the art (see, for example, Sargent, "Isolation of

20

30

35

Differentially Expressed Genes," in Meth. Enzymol. 152:423, 1987, and Wu et al. (eds.), "Construction and Screening of Subtracted and Complete Expression cDNA Libraries," in Methods in Gene Biotechnology, pages 29-65 (CRC Press, Inc. 1997)).

Various cloning vectors are appropriate for the construction of a cDNA library. For example, a cDNA library can be prepared in a vector derived from bacteriophage, such as a \(\lambda\gt10\) vector (see, for example, Huynh et al., "Constructing and Screening cDNA Libraries in Agt10 and Agt11," in DNA Cloning: A Practical Approach Vol. I, Glover (ed.), page 49 (IRL Press, 1985); Wu (1997) at pages 47-52).

Alternatively, double-stranded cDNA molecules can be inserted into a plasmid vector, such as a pBluescript vector (Stratagene Cloning Systems; La Jolla, California), a LambdaGEM-4 (Promega Corp.; Madison, Wisconsin) or other commercially available vectors. Suitable cloning vectors also can be obtained from the American Type Culture Collection (Rockville, Maryland).

In order to amplify the cloned cDNA molecules, the cDNA library is inserted into a prokaryotic host, using standard techniques. For example, a cDNA library 15 can be introduced into competent E. coli DH5 cells, which can be obtained from Life Technologies, Inc. (Gaithersburg, Maryland).

A human genomic DNA library can be prepared by means well-known in the art (see, for example, Ausubel (1995) at pages 5-1 to 5-6; Wu (1997) at pages 307-327). Genomic DNA can be isolated by lysing tissue with the detergent Sarkosyl, digesting the lysate with proteinase K, clearing insoluble debris from the lysate by centrifugation, precipitating nucleic acid from the lysate using isopropanol, and purifying resuspended DNA on a cesium chloride density gradient.

DNA fragments that are suitable for the production of a genomic library can be obtained by the random shearing of genomic DNA or by the partial digestion of genomic 25 DNA with restriction endonucleases. Genomic DNA fragments can be inserted into a vector, such as a bacteriophage or cosmid vector, in accordance with conventional techniques, such as the use of restriction enzyme digestion to provide appropriate termini, the use of alkaline phosphatase treatment to avoid undesirable joining of DNA molecules, and ligation with appropriate ligases. Techniques for such manipulation are well-known in the art (see, for example, Ausubel (1995) at pages 5-1 to 5-6; Wu (1997) at pages 307-327).

Nucleic acid molecules that encode a TGF-beta binding-protein gene can also be obtained using the polymerase chain reaction (PCR) with oligonucleotide primers having nucleotide sequences that are based upon the nucleotide sequences of the human TGF-beta binding-protein gene, as described herein. General methods for screening libraries with PCR are provided by, for example, Yu et al., "Use of the

25

Polymerase Chain Reaction to Screen Phage Libraries," in *Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications*, White (ed.), pages 211-215 (Humana Press, Inc. 1993). Moreover, techniques for using PCR to isolate related genes are described by, for example, Preston, "Use of Degenerate Oligonucleotide Primers and the Polymerase Chain Reaction to Clone Gene Family Members," in *Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications*, White (ed.), pages 317-337 (Humana Press, Inc. 1993).

Alternatively, human genomic libraries can be obtained from commercial sources such as Research Genetics (Huntsville, AL) and the American Type Culture Collection (Rockville, Maryland).

A library containing cDNA or genomic clones can be screened with one or more polynucleotide probes based upon SEQ ID NO:1, using standard methods (see, for example, Ausubel (1995) at pages 6-1 to 6-11).

Anti-TGF-beta binding-protein antibodies, produced as described below, can also be used to isolate DNA sequences that encode TGF-beta binding-protein genes from cDNA libraries. For example, the antibodies can be used to screen λgt11 expression libraries, or the antibodies can be used for immunoscreening following hybrid selection and translation (see, for example, Ausubel (1995) at pages 6-12 to 6-16; Margolis et al., "Screening λ expression libraries with antibody and protein probes," in DNA Cloning 2: Expression Systems, 2nd Edition, Glover et al. (eds.), pages 1-14 (Oxford University Press 1995)).

The sequence of a TGF-beta binding-protein cDNA or TGF-beta binding-protein genomic fragment can be determined using standard methods. Moreover, the identification of genomic fragments containing a TGF-beta binding-protein promoter or regulatory element can be achieved using well-established techniques, such as deletion analysis (see, generally, Ausubel (1995)).

As an alternative, a TGF-beta binding-protein gene can be obtained by synthesizing DNA molecules using mutually priming long oligonucleotides and the nucleotide sequences described herein (see, for example, Ausubel (1995) at pages 8-8 to 8-9). Established techniques using the polymerase chain reaction provide the ability to synthesize DNA molecules at least two kilobases in length (Adang et al., *Plant Molec. Biol. 21*:1131, 1993; Bambot et al., *PCR Methods and Applications 2*:266, 1993; Dillon et al., "Use of the Polymerase Chain Reaction for the Rapid Construction of Synthetic Genes," in *Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications*, White (ed.), pages 263-268, (Humana Press, Inc. 1993); Holowachuk et al., *PCR Methods Appl. 4*:299, 1995).

WO 00/32773

Production of TGF-beta binding-protein genes

Nucleic acid molecules encoding variant TGF-beta binding-protein genes can be obtained by screening various cDNA or genomic libraries with polynucleotide probes having nucleotide sequences based upon SEQ ID NO.1, 5, 9, 11, 13, or 15, using procedures described above. TGF-beta binding-protein gene variants can also be constructed synthetically. For example, a nucleic acid molecule can be devised that encodes a polypeptide having a conservative amino acid change, compared with the amino acid sequence of SEQ ID NOs: 2, 6, 8, 10, 12, 14, or 16. That is, variants can be obtained that contain one or more amino acid substitutions of SEQ ID NOs: 2, 6, 8, 10, 12, 14 or 16, in which an alkyl amino acid is substituted for an alkyl amino acid in a TGF-beta binding-protein amino acid sequence, an aromatic amino acid is substituted for an aromatic amino acid in a TGF-beta binding-protein amino acid sequence, a sulfur-containing amino acid is substituted for a sulfur-containing amino acid in a TGF-beta binding-protein amino acid sequence, a hydroxy-containing amino acid is substituted for a hydroxy-containing amino acid in a TGF-beta binding-protein amino acid sequence, an acidic amino acid is substituted for an acidic amino acid in a TGF-beta binding-protein amino acid sequence, a basic amino acid is substituted for a basic amino acid in a TGF-beta binding-protein amino acid sequence, or a dibasic monocarboxylic amino acid is substituted for a dibasic monocarboxylic amino acid in a TGF-beta binding-protein amino acid sequence.

Among the common amino acids, for example, a "conservative amino acid substitution" is illustrated by a substitution among amino acids within each of the following groups: (1) glycine, alanine, valine, leucine, and isoleucine, (2) phenylalanine, tyrosine, and tryptophan, (3) serine and threonine, (4) aspartate and glutamate, (5) glutamine and asparagine, and (6) lysine, arginine and histidine. In making such substitutions, it is important to, where possible, maintain the cysteine backbone outlined in Figure 1.

Conservative amino acid changes in a TGF-beta binding-protein gene can be introduced by substituting nucleotides for the nucleotides recited in SEQ ID NO:1. Such "conservative amino acid" variants can be obtained, for example, by oligonucleotide-directed mutagenesis, linker-scanning mutagenesis, mutagenesis using the polymerase chain reaction, and the like (see Ausubel (1995) at pages 8-10 to 8-22; and McPherson (ed.), Directed Mutagenesis: A Practical Approach (IRL Press 1991)). The functional ability of such variants can be determined using a standard method, such as the assay described herein. Alternatively, a variant TGF-beta binding-protein polypeptide can be identified by the ability to specifically bind anti-TGF-beta binding-

15

20

25

30

15

20

protein antibodies.

Routine deletion analyses of nucleic acid molecules can be performed to obtain "functional fragments" of a nucleic acid molecule that encodes a TGF-beta binding-protein polypeptide. As an illustration, DNA molecules having the nucleotide sequence of SEQ ID NO:1 can be digested with *Bal3*1 nuclease to obtain a series of nested deletions. The fragments are then inserted into expression vectors in proper reading frame, and the expressed polypeptides are isolated and tested for activity, or for the ability to bind anti-TGF-beta binding-protein antibodies. One alternative to exonuclease digestion is to use oligonucleotide-directed mutagenesis to introduce deletions or stop codons to specify production of a desired fragment. Alternatively, particular fragments of a TGF-beta binding-protein gene can be synthesized using the polymerase chain reaction.

Standard techniques for functional analysis of proteins are described by, for example, Treuter et al., *Molec. Gen. Genet.* 240:113, 1993; Content et al., "Expression and preliminary deletion analysis of the 42 kDa 2-5A synthetase induced by human interferon," in *Biological Interferon Systems, Proceedings of ISIR-TNO Meeting on Interferon Systems*, Cantell (ed.), pages 65-72 (Nijhoff 1987); Herschman, "The EGF Receptor," in *Control of Animal Cell Proliferation, Vol. 1*, Boynton et al., (eds.) pages 169-199 (Academic Press 1985); Coumailleau et al., *J. Biol. Chem.* 270:29270, 1995; Fukunaga et al., *J. Biol. Chem.* 270:25291, 1995; Yamaguchi et al., *Biochem. Pharmacol.* 50:1295, 1995; and Meisel et al., *Plant Molec. Biol.* 30:1, 1996.

The present invention also contemplates functional fragments of a TGF-beta binding-protein gene that have conservative amino acid changes.

A TGF-beta binding-protein variant gene can be identified on the basis of structure by determining the level of identity with nucleotide and amino acid 25 sequences of SEQ ID NOs: 1, 5, 9, 11, 13, or, 15 and 2, 6, 10, 12, 14, or 16, as discussed above. An alternative approach to identifying a variant gene on the basis of structure is to determine whether a nucleic acid molecule encoding a potential variant TGF-beta binding-protein gene can hybridize under stringent conditions to a nucleic acid molecule having the nucleotide sequence of SEQ ID Nos: 1, 5, 9, 11, 13, or, 15, or 30 a portion thereof of at least 15 or 20 nucleotides in length. As an illustration of stringent hybridization conditions, a nucleic acid molecule having a variant TGF-beta binding-protein sequence can bind with a fragment of a nucleic acid molecule having a sequence from SEQ ID NO:1 in a buffer containing, for example, 5xSSPE (1xSSPE = 180 mM sodium chloride, 10 mM sodium phosphate, 1 mM EDTA (pH 7.7), 35 5xDenhardt's solution (100xDenhardt's = 2% (w/v) bovine serum albumin, 2% (w/v)

10

15

20

25

30

35

Ficoll, 2% (w/v) polyvinylpyrrolidone) and 0.5% SDS incubated overnight at 55-60°C. Post-hybridization washes at high stringency are typically performed in 0.5xSSC (1xSSC = 150 mM sodium chloride, 15 mM trisodium citrate) or in 0.5xSSPE at 55-60°C.

Regardless of the particular nucleotide sequence of a variant TGF-beta binding-protein gene, the gene encodes a polypeptide that can be characterized by its functional activity, or by the ability to bind specifically to an anti-TGF-beta binding-protein antibody. More specifically, variant TGF-beta binding-protein genes encode polypeptides which exhibit at least 50%, and preferably, greater than 60, 70, 80 or 90%, of the activity of polypeptides encoded by the human TGF-beta binding-protein gene described herein.

4. Production of TGF-beta binding-protein in Cultured Cells

To express a TGF-beta binding-protein gene, a nucleic acid molecule encoding the polypeptide must be operably linked to regulatory sequences that control transcriptional expression in an expression vector and then introduced into a host cell. In addition to transcriptional regulatory sequences, such as promoters and enhancers, expression vectors can include translational regulatory sequences and a marker gene which is suitable for selection of cells that carry the expression vector.

Expression vectors that are suitable for production of a foreign protein in eukaryotic cells typically contain (1) prokaryotic DNA elements coding for a bacterial replication origin and an antibiotic resistance marker to provide for the growth and selection of the expression vector in a bacterial host; (2) eukaryotic DNA elements that control initiation of transcription, such as a promoter; and (3) DNA elements that control the processing of transcripts, such as a transcription termination/polyadenylation sequence.

TGF-beta binding-proteins of the present invention are preferably expressed in mammalian cells. Examples of mammalian host cells include African green monkey kidney cells (Vero; ATCC CRL 1587), human embryonic kidney cells (293-HEK; ATCC CRL 1573), baby hamster kidney cells (BHK-21; ATCC CRL 8544), canine kidney cells (MDCK; ATCC CCL 34), Chinese hamster ovary cells (CHO-K1; ATCC CCL61), rat pituitary cells (GH1; ATCC CCL82), HeLa S3 cells (ATCC CCL2.2), rat hepatoma cells (H-4-II-E; ATCC CRL 1548) SV40-transformed monkey kidney cells (COS-1; ATCC CRL 1650) and murine embryonic cells (NIH-3T3; ATCC CRL 1658).

For a mammalian host, the transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, simian virus, or the like, in which the regulatory signals are associated with a particular gene

which has a high level of expression. Suitable transcriptional and translational regulatory sequences also can be obtained from mammalian genes, such as actin, collagen, myosin, and metallothionein genes.

Transcriptional regulatory sequences include a promoter region sufficient to direct the initiation of RNA synthesis. Suitable eukaryotic promoters include the promoter of the mouse metallothionein I gene [Hamer et al., *J. Molec. Appl. Genet. 1*:273, 1982], the *TK* promoter of *Herpes* virus [McKnight, *Cell 31*:355, 1982], the *SI'40* early promoter [Benoist et al., *Nature 290*:304, 1981], the *Rous* sarcoma virus promoter [Gorman et al., *Proc. Nat'l Acad. Sci. USA 79*:6777, 1982], the cytomegalovirus promoter [Foecking et al., Gene 45:101, 1980], and the mouse mammary tumor virus promoter (see, generally, Etcheverry, "Expression of Engineered Proteins in Mammalian Cell Culture," in *Protein Engineering: Principles and Practice*, Cleland et al. (eds.), pages 163-181 (John Wiley & Sons, Inc. 1996)).

Alternatively, a prokaryotic promoter, such as the bacteriophage T3 RNA polymerase promoter, can be used to control TGF-beta binding-protein gene expression in mammalian cells if the prokaryotic promoter is regulated by a eukaryotic promoter (Zhou et al., *Mol. Cell. Biol. 10*:4529, 1990; Kaufman et al., *Nucl. Acids Res. 19*:4485, 1991).

TGF-beta binding-protein genes may also be expressed in bacterial, yeast, insect, or plant cells. Suitable promoters that can be used to express TGF-beta binding-protein polypeptides in a prokaryotic host are well-known to those of skill in the art and include promoters capable of recognizing the T4, T3, Sp6 and T7 polymerases, the P_R and P_L promoters of bacteriophage lambda, the *trp*, *recA*, heat shock, *lacUV5*, *tac*, *lpp-lacSpr*, *phoA*, and *lacZ* promoters of *E. coli*, promoters of *B. subtilis*, the promoters of the bacteriophages of *Bacillus*, *Streptomyces* promoters, the *int* promoter of bacteriophage lambda, the *bla* promoter of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene. Prokaryotic promoters have been reviewed by Glick, *J. Ind. Microbiol. 1*:277, 1987, Watson et al., *Molecular Biology of the Gene*, 4th Ed. (Benjamin Cummins 1987), and by Ausubel et al. (1995)

Preferred prokaryotic hosts include E. coli and Bacillus subtilus.

Suitable strains of E. coli include BL21(DE3), BL21(DE3)pLysS, BL21(DE3)pLysE, DH1, DH4I, DH5, DH5I, DH5IF', DH5IMCR, DH10B, DH10B/p3, DH11S, C600, HB101, JM101, JM105, JM109, JM110, K38, RR1, Y1088, Y1089, CSH18, ER1451, and ER1647 (see, for example, Brown (Ed.), Molecular Biology Labfax (Academic Press 1991)). Suitable strains of Bacillus subtilus include BR151, YB886, M1119, M1120, and B170 (see, for example, Hardy, "Bacillus Cloning Methods," in DNA Cloning: A Practical Approach, Glover (Ed.) (IRL Press 1985)).

Methods for expressing proteins in prokaryotic hosts are well-known to those of skill in the art (see, for example, Williams et al., "Expression of foreign proteins in E. coli using plasmid vectors and purification of specific polyclonal antibodies," in DNA Cloning 2: Expression Systems, 2nd Edition, Glover et al. (eds.), page 15 (Oxford University Press 1995); Ward et al., "Genetic Manipulation and Expression of Antibodies," in Monoclonal Antibodies: Principles and Applications, page 137 (Wiley-Liss, Inc. 1995); and Georgiou, "Expression of Proteins in Bacteria," in Protein Engineering: Principles and Practice, Cleland et al. (eds.), page 101 (John Wiley & Sons, Inc. 1996)).

The baculovirus system provides an efficient means to introduce cloned 10 TGF-heta hinding-protein genes into insect cells. Suitable expression vectors are based upon the Autographa californica multiple nuclear polyhedrosis virus (AcMNPV), and contain well-known promoters such as Drosophila heat shock protein (hsp) 70 promoter, Autographa californica nuclear polyhedrosis virus immediate-early gene promoter (ie-1) and the delayed early 39K promoter, baculovirus p10 promoter, and the Drosophila metallothionein promoter. Suitable insect host cells include cell lines derived from IPLB-Sf-21, a Spodoptera frugiperda pupal ovarian cell line, such as Sf9 (ATCC CRL 1711), Sf21AE, and Sf21 (Invitrogen Corporation; San Diego, CA), as well as Drosophila Schneider-2 cells. Established techniques for producing recombinant proteins in baculovirus systems are provided by Bailey et al., "Manipulation of Baculovirus Vectors," in Methods in Molecular Biology, Volume 7: Gene Transfer and Expression Protocols, Murray (ed.), pages 147-168 (The Humana Press, Inc. 1991), by Patel et al., "The baculovirus expression system," in DNA Cloning 2: Expression Systems, 2nd Edition, Glover et al. (eds.), pages 205-244 (Oxford University Press 1995), by Ausubel (1995) at pages 16-37 to 16-57, by Richardson (ed.), Baculovirus Expression Protocols (The Humana Press, Inc. 1995), and by Lucknow, "Insect Cell Expression Technology," in Protein Engineering: Principles and Practice, Cleland et al. (eds.), pages 183-218 (John Wiley & Sons, Inc. 1996).

Promoters for expression in yeast include promoters from GAL1 (galactose), PGK (phosphoglycerate kinase), ADH (alcohol dehydrogenase), AOX1 30 (alcohol oxidase), HIS4 (histidinol dehydrogenase), and the like. Many yeast cloning vectors have been designed and are readily available. These vectors include YIp-based vectors, such as YIp5, YRp vectors, such as YRp17, YEp vectors such as YEp13 and YCp vectors, such as YCp19. One skilled in the art will appreciate that there are a wide variety of suitable vectors for expression in yeast cells. 35

Expression vectors can also be introduced into plant protoplasts, intact plant

20

10

tissues, or isolated plant cells. General methods of culturing plant tissues are provided, for example, by Miki et al., "Procedures for Introducing Foreign DNA into Plants," in *Methods in Plant Molecular Biology and Biotechnology*, Glick et al. (eds.), pages 67-88 (CRC Press, 1993).

An expression vector can be introduced into host cells using a variety of standard techniques including calcium phosphate transfection, liposome-mediated transfection, microprojectile-mediated delivery, electroporation, and the like. Preferably, the transfected cells are selected and propagated to provide recombinant host cells that comprise the expression vector stably integrated in the host cell genome. Techniques for introducing vectors into eukaryotic cells and techniques for selecting such stable transformants using a dominant selectable marker are described, for example, by Ausubel (1995) and by Murray (ed.), *Gene Transfer and Expression Protocols* (Humana Press 1991). Methods for introducing expression vectors into bacterial, yeast, insect, and plant cells are also provided by Ausubel (1995).

General methods for expressing and recovering foreign protein produced by a mammalian cell system is provided by, for example, Etcheverry, "Expression of Engineered Proteins in Mammalian Cell Culture," in *Protein Engineering: Principles and Practice*, Cleland et al. (eds.), pages 163 (Wiley-Liss, Inc. 1996). Standard techniques for recovering protein produced by a bacterial system is provided by, for example, Grisshammer et al., "Purification of over-produced proteins from E. coli cells," in DNA Cloning 2: Expression Systems, 2nd Edition, Glover et al. (eds.), pages 59-92 (Oxford University Press 1995). Established methods for isolating recombinant proteins from a baculovirus system are described by Richardson (ed.), Baculovirus Expression Protocols (The Humana Press, Inc., 1995).

More generally, TGF-beta binding-protein can be isolated by standard techniques, such as affinity chromatography, size exclusion chromatography, ion exchange chromatography, HPLC and the like. Additional variations in TGF-beta binding-protein isolation and purification can be devised by those of skill in the art. For example, anti-TGF-beta binding-protein antibodies, obtained as described below, can be used to isolate large quantities of protein by immunoaffinity purification.

5. Production of Antibodies to TGF-beta binding-proteins

Antibodies to TGF-beta binding-protein can be obtained, for example, using the product of an expression vector as an antigen. Particularly useful anti-TGF-beta binding-protein antibodies "bind specifically" with TGF-beta binding-protein of Sequence ID Nos. 2, 6, 10, 12, 14, or 16, but not to other TGF-beta binding-proteisn

such as Dan, Cerberus, SCGF, or Gremlin. Antibodies of the present invention (including fragments and derivatives thereof) may be a polyclonal or, especially a monoclonal antibody. The antibody may belong to any immunoglobulin class, and may be for example an IgG, for example IgG₁, IgG₂, IgG₃, IgG₄, IgE; IgM; or IgA antibody. It may be of animal, for example mammalian origin, and may be for example a murine, rat, human or other primate antibody. Where desired the antibody may be an internalising antibody.

Polyclonal antibodies to recombinant TGF-beta binding-protein can be prepared using methods well-known to those of skill in the art (see, for example, Green et al., "Production of Polyclonal Antisera," in *Immunochemical Protocols* (Manson, ed.), pages 1-5 (Humana Press 1992); Williams et al., "Expression of foreign proteins in *E. coli* using plasmid vectors and purification of specific polyclonal antibodies," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover et al. (eds.), page 15 (Oxford University Press 1995)). Although polyclonal antibodies are typically raised in animals such as rats, mice, rabbits, goats, or sheep, an anti-TGF-beta binding-protein antibody of the present invention may also be derived from a subhuman primate antibody. General techniques for raising diagnostically and therapeutically useful antibodies in baboons may be found, for example, in Goldenberg et al., international patent publication No. WO 91/11465 (1991), and in Losman et al., *Int. J. Cancer 46*:310, 1990.

The antibody should comprise at least a variable region domain. The variable region domain may be of any size or amino acid composition and will generally comprise at least one hypervariable amino acid sequence responsible for antigen binding embedded in a framework sequence. In general terms the variable (V) region domain may be any suitable arrangement of immunoglobulin heavy (V_H) and/or light (V_L) chain variable domains. Thus for example the V region domain may be monomeric and be a V_H or V_L domain where these are capable of independently binding antigen with acceptable affinity. Alternatively the V region domain may be dimeric and contain $V_{H^-}V_H$, $V_{H^-}V_L$, or $V_L^-V_L$, dimers in which the V_H and V_L chains are non-covalently associated (abbreviated hereinafter as F_V). Where desired, however, the chains may be covalently coupled either directly, for example via a disulphide bond between the two variable domains, or through a linker, for example a peptide linker, to form a single chain domain (abbreviated hereinafter as scF_V).

The variable region domain may be any naturally occurring variable domain or an engineered version thereof. By engineered version is meant a variable region domain which has been created using recombinant DNA engineering techniques. Such engineered

15

20

versions include those created for example from natural antibody variable regions by insertions, deletions or changes in or to the amino acid sequences of the natural antibodies. Particular examples of this type include those engineered variable region domains containing at least one CDR and optionally one or more framework amino acids from one antibody and the remainder of the variable region domain from a second antibody.

The variable region domain may be covalently attached at a C-terminal amino acid to at least one other antibody domain or a fragment thereof. Thus, for example where a V_H domain is present in the variable region domain this may be linked to an immunoglobulin C_H 1 domain or a fragment thereof. Similarly a V_L domain may be linked to a C_K domain or a fragment thereof. In this way for example the antibody may be a Fab fragment wherein the antigen binding domain contains associated V_H and V_L domains covalently linked at their C-termini to a CH1 and C_K domain respectively. The CH1 domain may be extended with further amino acids, for example to provide a hinge region domain as found in a Fab' fragment, or to provide further domains, such as antibody CH2 and CH3 domains.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells (see, for example, Larrick et al., *Methods: A Companion to Methods in Enzymology 2:*106, 1991; Courtenay-Luck, "Genetic Manipulation of Monoclonal Antibodies," in *Monoclonal Antibodies: Production, Engineering and Clinical Application*, Ritter et al. (eds.), page 166 (Cambridge University Press 1995); and Ward et al., "Genetic Manipulation and Expression of Antibodies," in *Monoclonal Antibodies: Principles and Applications*, Birch et al., (eds.), page 137 (Wiley-Liss, Inc. 1995)).

Antibodies for use in the invention may in general be monoclonal (prepared by conventional immunisation and cell fusion procedures) or in the case of fragments, derived therefrom using any suitable standard chemical e.g. reduction or enzymatic cleavage and/or digestion techniques, for example by treatment with pepsin.

More specifically, monoclonal anti-TGF-beta binding-protein antibodies can be generated utilizing a variety of techniques. Rodent monoclonal antibodies to specific antigens may be obtained by methods known to those skilled in the art (see, for example, Kohler et al., *Nature 256*:495, 1975; and Coligan et al. (eds.), *Current Protocols in Immunology*, 1:2.5.1-2.6.7 (John Wiley & Sons 1991) ["Coligan"]; Picksley et al., "Production of monoclonal antibodies against proteins expressed in *E*.

15

25

30

10

20

25

30

35

coli," in DNA Cloning 2: Expression Systems, 2nd Edition, Glover et al. (eds.), page 93 (Oxford University Press 1995)).

Briefly, monoclonal antibodies can be obtained by injecting mice with a composition comprising a TGF-beta binding-protein gene product, verifying the presence of antibody production by removing a serum sample, removing the spleen to obtain B-lymphocytes, fusing the B-lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones which produce antibodies to the antigen, culturing the clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures.

In addition, an anti-TGF-beta binding-protein antibody of the present invention may be derived from a human monoclonal antibody. Human monoclonal antibodies are obtained from transgenic mice that have been engineered to produce specific human antibodies in response to antigenic challenge. In this technique, elements of the human heavy and light chain locus are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the endogenous heavy chain and light chain loci. The transgenic mice can synthesize human antibodies specific for human antigens, and the mice can be used to produce human antibody-secreting hybridomas. Methods for obtaining human antibodies from transgenic mice are described, for example, by Green et al., *Nature Genet.* 7:13, 1994; Lonberg et al., *Nature 368*:856, 1994; and Taylor et al., *Int. Immun. 6*:579, 1994.

Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography (see, for example, Coligan at pages 2.7.1-2.7.12 and pages 2.9.1-2.9.3; Baines et al., "Purification of Immunoglobulin G (IgG)," in Methods in Molecular Biology, Vol. 10, pages 79-104 (The Humana Press, Inc. 1992)).

For particular uses, it may be desirable to prepare fragments of anti-TGF-beta binding-protein antibodies. Such antibody fragments can be obtained, for example, by proteolytic hydrolysis of the antibody. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. As an illustration, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab')₂. This fragment can be further cleaved using a thiol reducing agent to produce 3.5S Fab' monovalent fragments. Optionally, the cleavage reaction can be performed using a blocking group for the sulfhydryl groups that result from cleavage of disulfide linkages. As an alternative, an enzymatic cleavage using pepsin produces two monovalent Fab

20

25

fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. patent No. 4,331,647, Nisonoff et al., Arch Biochem. Biophys. 89:230, 1960, Porter, Biochem. J. 73:119, 1959, Edelman et al., in Methods in Enzymology 1:422 (Academic Press 1967), and by Coligan at pages 2.8.1-2.8.10 and 2.10.-2.10.4.

Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

Alternatively, the antibody may be a recombinant or engineered antibody obtained by the use of recombinant DNA techniques involving the manipulation and reexpression of DNA encoding antibody variable and/or constant regions. Such DNA is known and/or is readily available from DNA libraries including for example phage-antibody libraries (see Chiswell, D J and McCafferty, J. Tibtech. 10 80-84 (1992)) or where desired can be synthesised. Standard molecular biology and/or chemistry procedures may be used to sequence and manipulate the DNA, for example, to introduce codons to create cysteine residues, to modify, add or delete other amino acids or domains as desired.

From here, one or more replicable expression vectors containing the DNA may be prepared and used to transform an appropriate cell line, e.g. a non-producing myeloma cell line, such as a mouse NSO line or a bacterial, e.g. E.coli line, in which production of the antibody will occur. In order to obtain efficient transcription and translation, the DNA sequence in each vector should include appropriate regulatory sequences, particularly a promoter and leader sequence operably linked to the variable domain sequence. Particular methods for producing antibodies in this way are generally well known and routinely used. For example, basic molecular biology procedures are described by Maniatis et al (Molecular Cloning, Cold Spring Harbor Laboratory, New York, 1989); DNA sequencing can be performed as described in Sanger et al (PNAS 74, 5463, (1977)) and the Amersham International plc sequencing handbook; and site directed mutagenesis can be carried out according to the method of Kramer et al (Nucl. Acids Res. 12, 9441, (1984)) and the Anglian Biotechnology Ltd handbook. Additionally, there are numerous publications, detailing techniques suitable for the preparation of antibodies by manipulation of DNA, creation of expression vectors and transformation of appropriate cells, for example as reviewed by Mountain A and Adair, J R in Biotechnology and Genetic Engineering Reviews (ed. Tombs, M P, 10, Chapter 1, 1992, Intercept, Andover, UK) and in International Patent Specification No. WO 91/09967.

Where desired, the antibody according to the invention may have one or

15

more effector or reporter molecules attached to it and the invention extends to such modified proteins. The effector or reporter molecules may be attached to the antibody through any available amino acid side-chain, terminal amino acid or, where present carbohydrate functional group located in the antibody, always provided of course that this does not adversely affect the binding properties and eventual usefulness of the molecule. Particular functional groups include, for example any free amino, imino, thiol, hydroxyl, carboxyl or aldehyde group. Attachment of the antibody and the effector and/or reporter molecule(s) may be achieved via such groups and an appropriate functional group in the effector or reporter molecules. The linkage may be direct or indirect, through spacing or bridging groups.

Effector molecules include, for example, antineoplastic agents, toxins (such as enzymatically active toxins of bacterial or plant origin and fragments thereof e.g. ricin and fragments thereof) biologically active proteins, for example enzymes, nucleic acids and fragments thereof, e.g. DNA, RNA and fragments thereof, naturally occurring and synthetic polymers e.g. polysaccharides and polyalkylene polymers such as poly(ethylene glycol) and derivatives thereof, radionuclides, particularly radioiodide, and chelated metals. Suitable reporter groups include chelated metals, fluorescent compounds or compounds which may be detected by NMR or ESR spectroscopy.

Particular antineoplastic agents include cytotoxic and cytostatic agents, for example alkylating agents, such as nitrogen mustards (e.g. chlorambucil, melphalan, 20 mechlorethamine, cyclophosphamide, or uracil mustard) and derivatives thereof, triethylenephosphoramide, triethylenethiophosphor-amide, busulphan, antimetabolites, such as methotrexate, fluorouracil, floxuridine, cytarabine, mercaptopurine, thioguanine, fluoroacetic acid or fluorocitric acid, antibiotics, such as bleomycins (e.g. bleomycin sulphate), doxorubicin, daunorubicin, mitomycins (e.g. mitomycin C), 25 actinomycins (e.g. dactinomycin) plicamycin, calichaemicin and derivatives thereof, or esperamicin and derivatives thereof; mitotic inhibitors, such as etoposide, vincristine or vinblastine and derivatives thereof; alkaloids, such as ellipticine; polyols such as taxicin-l or taxicin-II; hormones, such as androgens (e.g. dromostanolone or testolactone), progestins (e.g. megestrol acetate or medroxyprogesterone acetate), estrogens (e.g. dimethylstilbestrol 30 diphosphate, polyestradiol phosphate or estramustine phosphate) or antiestrogens (e.g. tamoxifen); anthraquinones, such as mitoxantrone, ureas, such as hydroxyurea; hydrazines, such as procarbazine; or imidazoles, such as dacarbazine.

Particularly useful effector groups are calichaemicin and derivatives thereof (see for example South African Patent Specifications Nos. 85/8794, 88/8127 and 90/2839).

Chelated metals include chelates of di-or tripositive metals having a

20

25

coordination number from 2 to 8 inclusive. Particular examples of such metals include technetium (Tc), rhenium (Re), cobalt (Co), copper (Cu), gold (Au), silver (Ag), lead (Pb), bismuth (Bi), indium (In), gallium (Ga), yttrium (Y), terbium (Tb), gadolinium (Gd), and scandium (Sc). In general the metal is preferably a radionuclide. Particular radionuclides include ^{99m}Tc, ¹⁸⁶Re, ⁵⁸Co, ⁶⁰Co, ⁶⁷Cu, ¹⁹⁵Au, ¹⁹⁹Au, ¹¹⁰Ag, ²⁰³Pb, ²⁰⁶Bi, ²⁰⁷Bi, ¹¹¹In, ⁶⁷Ga, ⁶⁸Ga, ⁸⁸Y, ⁹⁰Y, ¹⁶⁰Tb, ¹⁵³Gd and ⁴⁷Sc.

The chelated metal may be for example one of the above types of metal chelated with any suitable polydentate chelating agent, for example acyclic or cyclic polyamines, polyethers, (e.g. crown ethers and derivatives thereof), polyamides, porphyrins, and carbocyclic derivatives.

In general, the type of chelating agent will depend on the metal in use. One particularly useful group of chelating agents in conjugates according to the invention, however, are acyclic and cyclic polyamines, especially polyaminocarboxylic acids, for example diethylenetriaminepentaacetic acid and derivatives thereof, and macrocyclic amines, e.g. cyclic tri-aza and tetra-aza derivatives (for example as described in International Patent Specification No. WO 92/22583); and polyamides, especially desferrioxamine and derivatives thereof.

Thus for example when it is desired to use a thiol group in the antibody as the point of attachment this may be achieved through reaction with a thiol reactive group present in the effector or reporter molecule. Examples of such groups include an á-halocarboxylic acid or ester, e.g. iodoacetamide, an imide, e.g. maleimide, a vinyl sulphone, or a disulphide. These and other suitable linking procedures are generally and more particularly described in International Patent Specifications Nos. WO 93/06231, WO 92/22583, WO 90/091195 and WO 89/01476.

ASSAYS FOR SELECTING MOLECULES WHICH INCREASE BONE DENSITY

As discussed above, the present invention provides methods for selecting and/or isolating compounds which are capable of increasing bone density. For example, within one aspect of the present invention methods are provided for determining whether a selected molecule is capable of increasing bone mineral content, comprising the steps of (a) mixing a selected molecule with TGF-beta binding protein and a selected member of the TGF-beta family of proteins, (b) determining whether the selected molecule stimulates signaling by the TGF-beta family of proteins, or inhibits the binding of the TGF-beta binding protein to the TGF-beta family of proteins. Within certain embodiments, the molecule enhances the ability of TGF-beta to function as a positive regulator of mesenchymal cell differentiation.

Within other aspects of the invention, methods are provided for determining whether a selected molecule is capable of increasing bone mineral content, comprising the steps of (a) exposing a selected molecule to cells which express TGF-beta binding-protein and (b) determining whether the expression (or activity) of TGF-beta binding-protein from said exposed cells decreases, and therefrom determining whether the compound is capable of increasing bone mineral content. Within one embodiment, the cells are selected from the group consisting of the spontaneously transformed or untransformed normal human bone from bone biopsies and rat parietal bone osteoblasts. Such methods may be accomplished in a wide variety of assay formats including, for example, Countercurrent Immuno-Electrophoresis (CIEP), Radioimmunoassays, Radioimmunoprecipitations, Enzyme-Linked Immuno-Sorbent Assays (ELISA), Dot Blot assays, Inhibition or Competition assays, and sandwich assays (see U.S. Patent Nos. 4,376,110 and 4,486,530; see also Antibodies: A Laboratory Manual, supra).

Representative embodiments of such assays are provided below in Examples 5 and 6. Briefly, a family member of the TGF-beta super-family or a TGF-beta binding protein is first bound to a solid phase, followed by addition of a candidate molecule. The labeled family member of the TGF-beta super-family or a TGF-beta binding protein is then added to the assay, the solid phase washed, and the quantity of bound or labeled TGF-beta super-family member or TGF-beta binding protein on the solid support determined. Molecules which are suitable for use in increasing bone mineral content as described herein are those molecules which decrease the binding of TGF-beta binding protein to a member or members of the TGF-beta super-family in a statistically significant manner. Obviously, assays suitable for use within the present invention should not be limited to the embodiments described within Examples 2 and 3. In particular, numerous parameters may be altered, such as by binding TGF-beta to a solid phase, or by elimination of a solid phase entirely.

Within other aspects of the invention, methods are provided for determining whether a selected molecule is capable of increasing bone mineral content, comprising the steps of (a) exposing a selected molecule to cells which express TGF-beta and (b) determining whether the activity of TGF-beta from said exposed cells is altered, and therefrom determining whether the compound is capable of increasing bone mineral content. Similar to the above described methods, a wide variety of methods may be utilized to assess the changes of TGF-beta binding-protein expression due to a selected test compound.

15

20

25

30

20

25

For example, within one aspect of the present invention methods are provided for determining whether a selected molecule is capable of increasing bone mineral content, comprising the steps of (a) mixing a selected molecule with TGF-betabinding-protein and a selected member of the TGF-beta family of proteins, (b) determining whether the selected molecule up-regulates the signaling of the TGFbeta family of proteins, or inhibits the binding of the TGF-beta binding-protein to the TGF-beta family of proteins. Within certain embodiments, the molecule enhances the ability of TGF-beta to function as a positive regulator of mechemchymal cell differentiation.

Similar to the above described methods, a wide variety of methods may be utilized to assess stimulation of TGF-beta due to a selected test compound. One such representative method is provided below in Example 6 (see also Durham et al., Endo. 136:1374-1380.

Within yet other aspects of the present invention, methods are provided for determining whether a selected molecule is capable of increasing bone mineral 15 content, comprising the step of determining whether a selected molecule inhibits the binding of TGF-beta binding-protein to bone, or an analogue thereof. As utilized herein, it should be understood that bone or analogues thereof refers to hydroxyapatite, or a surface composed of a powdered form of bone, crushed bone or intact bone. Similar to the above described methods, a wide variety of methods may be utilized to assess the inhibition of TGF-beta binding-protein localization to bone matrix. One such representative method is provided below in Example 7.

It should be noted that while the methods recited herein may refer to the analysis of an individual test molecule, that the present invention should not be so limited. In particular, the selected molecule may be contained within a mixture of compounds. Hence, the recited methods may further comprise the step of isolating a molecule which inhibits the binding of TGF-beta binding-protein to a TGF-beta family member

CANDIDATE MOLECULES

30 A wide variety of molecules may be assayed for their ability to inhibit the binding of TGF-beta binding-protein to a TGF-beta family member. Representative examples which are discussed in more detail below include organic molecules, proteins or peptides, and nucleic acid molecules. Although it should be evident from the discussion below that the candidate molecules described herein may be utilized in the

assays described herein, it should also be readily apparent that such molecules can also be utilized in a variety of diagnostic and therapeutic settins.

1. Organic Molecules

Numerous organic molecules may be assayed for their ability to inhibit the binding of TGF-beta binding-protein to a TGF-beta family member.

For example, within one embodiment of the invention suitable organic molecules may be selected from either a chemical library, wherein chemicals are assayed individually, or from combinatorial chemical libraries where multiple compounds are assayed at once, then deconvoluted to determine and isolate the most active compounds.

Representative examples of such combinatorial chemical libraries include those described by Agrafiotis et al., "System and method of automatically generating chemical compounds with desired properties," U.S. Patent No. 5,463,564; Armstrong, R.W., "Synthesis of combinatorial arrays of organic compounds through ... the use of multiple component combinatorial array syntheses," WO 95/02566; Baldwin, 15 J.J. et al., "Sulfonamide derivatives and their use," WO 95/24186; Baldwin, J.J. et al., "Combinatorial dihydrobenzopyran library," WO 95/30642; Brenner, S., "New kit for preparing combinatorial libraries," WO 95/16918; Chenera, B. et al., "Preparation of library of resin-bound aromatic carbocyclic compounds," WO 95/16712; Ellman, J.A., "Solid phase and combinatorial synthesis of benzodiazepine compounds on a solid support," U.S. Patent No. 5,288,514; Felder, E. et al., "Novel combinatorial compound libraries," WO 95/16209; Lerner, R. et al., "Encoded combinatorial chemical libraries," WO 93/20242; Pavia, M.R. et al., "A method for preparing and selecting pharmaceutically useful non-peptide compounds from a structurally diverse universal library," WO 95/04277; Summerton, J.E. and D.D. Weller, "Morpholino-subunit 25 combinatorial library and method," U.S. Patent No. 5,506,337; Holmes, C., "Methods for the Solid Phase Synthesis of Thiazolidinones, Metathiazanones, and Derivatives thereof," WO 96/00148; Phillips, G.B. and G.P. Wei, "Solid-phase Synthesis of Benzimidazoles," Tet. Letters 37:4887-90, 1996; Ruhland, B. et al., "Solid-supported Combinatorial Synthesis of Structurally Diverse \(\beta\)-Lactams," J. Amer. Chem. Soc. 30 111:253-4, 1996; Look, G.C. et al., "The Indentification of Cyclooxygenase-1 Inhibitors from 4-Thiazolidinone Combinatorial Libraries," Bioorg and Med. Chem. Letters 6:707-12, 1996.

10

2. Proteins and Peptides

A wide range of proteins and peptides may likewise be utilized as candidate molecules for inhibitors of the binding of TGF-beta binding-protein to a TGF-beta family member.

a. Combinatorial Peptide Libraries

Peptide molecules which are putative inhibitors of the binding of TGF-beta binding-protein to a TGF-beta family member may be obtained through the screening of combinatorial peptide libraries. Such libraries may either be prepared by one of skill in the art (see e.g., U.S. Patent Nos. 4,528,266 and 4,359,535, and Patent Cooperation Treaty Publication Nos. WO 92/15679, WO 92/15677, WO 90/07862, WO 90/02809, or purchased from commercially available sources (e.g., New England Biolabs Ph.D.TM Phage Display Peptide Library Kit).

b. Antibodies

Antibodies which inhibit the binding of TGF-beta binding-protein to a TGF-beta family member may readily be prepared given the disclosure provided herein. Within the context of the present invention, antibodies are understood to include monoclonal antibodies, polyclonal antibodies, anti-idiotypic antibodies, antibody fragments (e.g., Fab, and F(ab')₂, F_V variable regions, or complementarity determining regions). As discussed above, antibodies are understood to be specific against TGF-beta binding-protein, or against a specific TGF-beta family member, if they bind with a K_a of greater than or equal to 10⁷M, preferably greater than or equal to 10⁸M, and do not bind to other TGF-beta binding-proteins, or, bind with a K_a of less than or equal to 10⁶M. Furthermore, antibodies of the present invention should block or inhibit the binding of TGF-beta binding-protein to a TGF-beta family member.

The affinity of a monoclonal antibody or binding partner, as well as inhibition of binding can be readily determined by one of ordinary skill in the art (see Scatchard, Ann. N.Y. Acad. Sci. 51:660-672, 1949).

Briefly, polyclonal antibodies may be readily generated by one of ordinary skill in the art from a variety of warm-blooded animals such as horses, cows, various fowl, rabbits, mice, or rats. Typically, the TGF-beta binding-protein or unique peptide thereof of 13-20 amino acids (preferably conjugated to keyhole limpet hemocyanin by cross-linking with glutaraldehyde) is utilized to immunize the animal through intraperitoneal, intramuscular, intraocular, or subcutaneous injections, along with an adjuvant such as Freund's complete or incomplete adjuvant. Following several

15

20

25

30

35

booster immunizations, samples of serum are collected and tested for reactivity to the protein or peptide. Particularly preferred polyclonal antisera will give a signal on one of these assays that is at least three times greater than background. Once the titer of the animal has reached a plateau in terms of its reactivity to the protein, larger quantities of antisera may be readily obtained either by weekly bleedings, or by exsanguinating the animal.

Monoclonal antibodies may also be readily generated using conventional techniques (see U.S. Patent Nos. RE 32,011, 4,902,614, 4,543,439, and 4,411,993 which are incorporated herein by reference; see also Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses, Plenum Press, Kennett, McKearn, and Bechtol (eds.), 1980, and Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988, which are also incorporated herein by reference).

Briefly, within one embodiment a subject animal such as a rat or mouse is immunized with TGF-beta binding-protein or portion thereof as described above. The protein may be admixed with an adjuvant such as Freund's complete or incomplete adjuvant in order to increase the resultant immune response. Between one and three weeks after the initial immunization the animal may be reimmunized with another booster immunization, and tested for reactivity to the protein utilizing assays described above. Once the animal has reached a plateau in its reactivity to the injected protein, it is sacrificed, and organs which contain large numbers of B cells such as the spleen and lymph nodes are harvested.

Cells which are obtained from the immunized animal may be immortalized by infection with a virus such as the Epstein-Barr virus (EBV) (see Glasky and Reading, *Hybridoma 8*(4):377-389, 1989). Alternatively, within a preferred embodiment, the harvested spleen and/or lymph node cell suspensions are fused with a suitable myeloma cell in order to create a "hybridoma" which secretes monoclonal antibody. Suitable myeloma lines include, for example, NS-1 (ATCC No. TIB 18), and P3X63 - Ag 8.653 (ATCC No. CRL 1580).

Following the fusion, the cells may be placed into culture plates containing a suitable medium, such as RPMI 1640, or DMEM (Dulbecco's Modified Eagles Medium) (JRH Biosciences, Lenexa, Kansas), as well as additional ingredients, such as fetal bovine serum (FBS, *i.e.*, from Hyclone, Logan, Utah, or JRH Biosciences). Additionally, the medium should contain a reagent which selectively allows for the growth of fused spleen and myeloma cells such as HAT (hypoxanthine, aminopterin, and thymidine) (Sigma Chemical Co., St. Louis, Missouri). After about

10

15

20

30

seven days, the resulting fused cells or hybridomas may be screened in order to determine the presence of antibodies which are reactive against TGF-beta binding-protein (depending on the antigen used), and which block or inhibit the binding of TGF-beta binding-protein to a TGF-beta family member.

A wide variety of assays may be utilized to determine the presence of antibodies which are reactive against the proteins of the present invention, including for example countercurrent immuno-electrophoresis, radioimmunoassays, radioimmunoprecipitations, enzyme-linked immuno-sorbent assays (ELISA), dot blot assays, western blots, immunoprecipitation, inhibition or competition assays, and sandwich assays (see U.S. Patent Nos. 4,376,110 and 4,486,530; see also Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988). Following several clonal dilutions and reassays, a hybridoma producing antibodies reactive against the desired protein may be isolated.

Other techniques may also be utilized to construct monoclonal antibodies (see William D. Huse et al., "Generation of a Large Combinational Library of the Immunoglobulin Repertoire in Phage Lambda," Science 246:1275-1281, December 1989; see also L. Sastry et al., "Cloning of the Immunological Repertoire in Escherichia coli for Generation of Monoclonal Catalytic Antibodies: Construction of a Heavy Chain Variable Region-Specific cDNA Library," Proc. Natl. Acad. Sci. USA 86:5728-5732, August 1989; see also Michelle Alting-Mees et al., "Monoclonal Antibody Expression Libraries: A Rapid Alternative to Hybridomas," Strategies in Molecular Biology 3:1-9, January 1990). These references describe a commercial system available from Stratagene (La Jolla, California) which enables the production of antibodies through recombinant techniques. Briefly, mRNA is isolated from a B cell population, and utilized to create heavy and light chain immunoglobulin cDNA expression libraries in the $\lambda ImmunoZap(H)$ and $\lambda ImmunoZap(L)$ vectors. vectors may be screened individually or co-expressed to form Fab fragments or antibodies (see Huse et al., supra; see also Sastry et al., supra). Positive plaques may subsequently be converted to a non-lytic plasmid which allows high level expression of monoclonal antibody fragments from E. coli.

Similarly, portions or fragments, such as Fab and Fv fragments, of antibodies may also be constructed utilizing conventional enzymatic digestion or recombinant DNA techniques to incorporate the variable regions of a gene which encodes a specifically binding antibody. Within one embodiment, the genes which encode the variable region from a hybridoma producing a monoclonal antibody of interest are amplified using nucleotide primers for the variable region. These primers

may be synthesized by one of ordinary skill in the art, or may be purchased from commercially available sources. Stratagene (La Jolla, California) sells primers for mouse and human variable regions including, among others, primers for VHa, VHb, VHc, VHd, CH1, VL and CL regions. These primers may be utilized to amplify heavy or light chain variable regions, which may then be inserted into vectors such as ImmunoZAPTM H or ImmunoZAPTM L (Stratagene), respectively. These vectors may then be introduced into E. coli, yeast, or mammalian-based systems for expression. Utilizing these techniques, large amounts of a single-chain protein containing a fusion of the VH and VL domains may be produced (see Bird et al., Science 242:423-426, 1988). In addition, such techniques may be utilized to change a "murine" antibody to a "human" antibody, without altering the binding specificity of the antibody.

Once suitable antibodies have been obtained, they may be isolated or purified by many techniques well known to those of ordinary skill in the art (see Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988). Suitable techniques include peptide or protein affinity columns, HPLC or RP-HPLC, purification on protein A or protein G columns, or any combination of these techniques.

c. Mutant TGF-beta binding-proteins

As described herein and below in the Examples (e.g., Examples 8 and 9), altered versions of TGF-beta binding-protein which compete with native TGF-beta binding-protein's ability to block the activity of a particular TGF-beta family member should lead to increased bone density. Thus, mutants of TGF-beta binding-protein which bind to the TGF-beta family member but do not inhibit the function of the TGF-beta family member would meet the criteria. The mutant versions must effectively compete with the endogenous inhibitory functions of TGF-beta binding-protein.

d. Production of proteins

Although various genes (or portions thereof) have been provided herein, it should be understood that within the context of the present invention, reference to one or more of these genes includes derivatives of the genes that are substantially similar to the genes (and, where appropriate, the proteins (including peptides and polypeptides) that are encoded by the genes and their derivatives). As used herein, a nucleotide sequence is deemed to be "substantially similar" if: (a) the nucleotide sequence is derived from the coding region of the above-described genes and includes, for example, portions of the sequence or allelic variations of the sequences discussed

15

20

25

above, or alternatively, encodes a molecule which inhibits the binding of TGF-beta binding-protein to a member of the TGF-beta family, (b) the nucleotide sequence is capable of hybridization to nucleotide sequences of the present invention under moderate, high or very high stringency (see Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, NY, 1989); or (c) the DNA sequences are degenerate as a result of the genetic code to the DNA sequences defined in (a) or (b). Further, the nucleic acid molecule disclosed herein includes both complementary and non-complementary sequences, provided the sequences otherwise meet the criteria set forth herein. Within the context of the present invention, high stringency means standard hybridization conditions (e.g., 5XSSPE, 0.5% SDS at 65°C, or the equivalent).

The structure of the proteins encoded by the nucleic acid molecules described herein may be predicted from the primary translation products using the hydrophobicity plot function of, for example, P/C Gene or Intelligenetics Suite (Intelligenetics, Mountain View, California), or according to the methods described by Kyte and Doolittle (*J. Mol. Biol. 157*:105-132, 1982).

Proteins of the present invention may be prepared in the form of acidic or basic salts, or in neutral form. In addition, individual amino acid residues may be modified by oxidation or reduction. Furthermore, various substitutions, deletions, or additions may be made to the amino acid or nucleic acid sequences, the net effect of which is to retain or further enhance or decrease the biological activity of the mutant or wild-type protein. Moreover, due to degeneracy in the genetic code, for example, there may be considerable variation in nucleotide sequences encoding the same amino acid sequence.

Other derivatives of the proteins disclosed herein include conjugates of the proteins along with other proteins or polypeptides. This may be accomplished, for example, by the synthesis of N-terminal or C-terminal fusion proteins which may be added to facilitate purification or identification of proteins (see U.S. Patent No. 4,851,341, see also, Hopp et al., Bio/Technology 6:1204, 1988.) Alternatively, fusion proteins such as Flag/TGF-beta binding-protein be constructed in order to assist in the identification, expression, and analysis of the protein.

Proteins of the present invention may be constructed using a wide variety of techniques described herein. Further, mutations may be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following

ligation, the resulting reconstructed sequence encodes a derivative having the desired amino acid insertion, substitution, or deletion.

Alternatively, oligonucleotide-directed site-specific (or segment specific) mutagenesis procedures may be employed to provide an altered gene having particular codons altered according to the substitution, deletion, or insertion required. Exemplary methods of making the alterations set forth above are disclosed by Walder et al. (Gene 42:133, 1986); Bauer et al. (Gene 37:73, 1985); Craik (BioTechniques, January 1985, 12-19); Smith et al. (Genetic Engineering: Principles and Methods, Plenum Press, 1981); and Sambrook et al. (supra). Deletion or truncation derivatives of proteins (e.g., a soluble extracellular portion) may also be constructed by utilizing convenient restriction endonuclease sites adjacent to the desired deletion. Subsequent to restriction, overhangs may be filled in, and the DNA religated. Exemplary methods of making the alterations set forth above are disclosed by Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, 1989).

Mutations which are made in the nucleic acid molecules of the present invention preferably preserve the reading frame of the coding sequences. Furthermore, the mutations will preferably not create complementary regions that could hybridize to produce secondary mRNA structures, such as loops or hairpins, that would adversely affect translation of the mRNA. Although a mutation site may be predetermined, it is not necessary that the nature of the mutation *per se* be predetermined. For example, in order to select for optimum characteristics of mutants at a given site, random mutagenesis may be conducted at the target codon and the expressed mutants screened for indicative biological activity. Alternatively, mutations may be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes a derivative having the desired amino acid insertion, substitution, or deletion.

Nucleic acid molecules which encode proteins of the present invention may also be constructed utilizing techniques of PCR mutagenesis, chemical mutagenesis (Drinkwater and Klinedinst, *PNAS* 83:3402-3406, 1986), by forced nucleotide misincorporation (e.g., Liao and Wise Gene 88:107-111, 1990), or by use of randomly mutagenized oligonucleotides (Horwitz et al., Genome 3:112-117, 1989).

The present invention also provides for the manipulation and expression of the above described genes by culturing host cells containing a vector capable of expressing the above-described genes. Such vectors or vector constructs include either synthetic or cDNA-derived nucleic acid molecules encoding the desired protein, which

15

20

25

30

are operably linked to suitable transcriptional or translational regulatory elements. Suitable regulatory elements may be derived from a variety of sources, including bacterial, fungal, viral, mammalian, insect, or plant genes. Selection of appropriate regulatory elements is dependent on the host cell chosen, and may be readily accomplished by one of ordinary skill in the art. Examples of regulatory elements include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a transcriptional terminator, and a ribosomal binding sequence, including a translation initiation signal.

Nucleic acid molecules that encode any of the proteins described above may be readily expressed by a wide variety of prokaryotic and eukaryotic host cells, including bacterial, mammalian, yeast or other fungi, viral, insect, or plant cells. Methods for transforming or transfecting such cells to express foreign DNA are well known in the art (see, e.g., Itakura et al., U.S. Patent No. 4,704,362; Hinnen et al., Proc. Natl. Acad. Sci. USA 75:1929-1933, 1978; Murray et al., U.S. Patent No. 4,801,542; Upshall et al., U.S. Patent No. 4,935,349; Hagen et al., U.S. Patent No. 4,784,950; Axel et al., U.S. Patent No. 4,399,216; Goeddel et al., U.S. Patent No. 4,766,075; and Sambrook et al. Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, 1989; for plant cells see Czako and Marton, Plant Physiol. 104:1067-1071, 1994; and Paszkowski et al., Biotech. 24:387-392,

Bacterial host cells suitable for carrying out the present invention include $E.\ coli$, $B.\ subtilis$, $Salmonella\ typhimurium$, and various species within the genera Pseudomonas, Streptomyces, and Staphylococcus, as well as many other bacterial species well known to one of ordinary skill in the art. Representative examples of bacterial host cells include DH5 α (Stratagene, LaJolla, California).

Bacterial expression vectors preferably comprise a promoter which functions in the host cell, one or more selectable phenotypic markers, and a bacterial origin of replication. Representative promoters include the β-lactamase (penicillinase) and lactose promoter system (see Chang et al., Nature 275:615, 1978), the T7 RNA polymerase promoter (Studier et al., Meth. Enzymol. 185:60-89, 1990), the lambda promoter (Elvin et al., Gene 87:123-126, 1990), the trp promoter (Nichols and Yanofsky, Meth. in Enzymology 101:155, 1983) and the tac promoter (Russell et al., Gene 20:231, 1982). Representative selectable markers include various antibiotic resistance markers such as the kanamycin or ampicillin resistance genes. Many plasmids suitable for transforming host cells are well known in the art, including among others, pBR322 (see Bolivar et al., Gene 2:95, 1977), the pUC plasmids pUC18,

pUC19, pUC118, pUC119 (see Messing, Meth. in Enzymology 101:20-77, 1983 and Vieira and Messing, Gene 19:259-268, 1982), and pNH8A, pNH16a, pNH18a, and Bluescript M13 (Stratagene, La Jolla, California).

Yeast and fungi host cells suitable for carrying out the present invention include, among others, Saccharomyces pombe, Saccharomyces cerevisiae, the genera Pichia or Kluyveromyces and various species of the genus Aspergillus (McKnight et al., U.S. Patent No. 4,935,349). Suitable expression vectors for yeast and fungi include, among others, YCp50 (ATCC No. 37419) for yeast, and the amdS cloning vector pV3 (Turnbull, Bio/Technology 7:169, 1989), YRp7 (Struhl et al., Proc. Natl. Acad. Sci. USA 76:1035-1039, 1978), YEp13 (Broach et al., Gene 8:121-133, 1979), pJDB249 and pJDB219 (Beggs, Nature 275:104-108, 1978) and derivatives thereof.

Preferred promoters for use in yeast include promoters from yeast glycolytic genes (Hitzeman et al., J. Biol. Chem. 255:12073-12080, 1980; Alber and Kawasaki, J. Mol. Appl. Genet. 1:419-434, 1982) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals, Hollaender et al. (eds.), p. 355, Plenum, New York, 1982; Ammerer, Meth. Enzymol. 101:192-201, 1983). Examples of useful promoters for fungi vectors include those derived from Aspergillus nidulans glycolytic genes, such as the adh3 promoter (McKnight et al., EMBO J. 4:2093-2099, 1985). The expression units may also include a transcriptional terminator. An example of a suitable terminator is the adh3 terminator (McKnight et al., ibid., 1985).

As with bacterial vectors, the yeast vectors will generally include a selectable marker, which may be one of any number of genes that exhibit a dominant phenotype for which a phenotypic assay exists to enable transformants to be selected. Preferred selectable markers are those that complement host cell auxotrophy, provide antibiotic resistance or enable a cell to utilize specific carbon sources, and include *leu2* (Broach et al., *ibid.*), *ura3* (Botstein et al., *Gene 8*:17, 1979), or *his3* (Struhl et al., *ibid.*). Another suitable selectable marker is the *cat* gene, which confers chloramphenicol resistance on yeast cells.

Techniques for transforming fungi are well known in the literature, and have been described, for instance, by Beggs (*ihid.*), Hinnen et al. (*Proc. Natl. Acad. Sci. USA 75*:1929-1933, 1978), Yelton et al. (*Proc. Natl. Acad. Sci. USA 81*:1740-1747, 1984), and Russell (*Nature 301*:167-169, 1983). The genotype of the host cell may contain a genetic defect that is complemented by the selectable marker present on the expression vector. Choice of a particular host and selectable marker is well within the level of ordinary skill in the art.

30

15

25

30

35

Protocols for the transformation of yeast are also well known to those of ordinary skill in the art. For example, transformation may be readily accomplished either by preparation of spheroplasts of yeast with DNA (see Hinnen et al., PNAS USA 75:1929, 1978) or by treatment with alkaline salts such as LiCl (see Itoh et al., J. Bacteriology 153:163, 1983). Transformation of fungi may also be carried out using polyethylene glycol as described by Cullen et al. (Bio/Technology 5:369, 1987).

Viral vectors include those which comprise a promoter that directs the expression of an isolated nucleic acid molecule that encodes a desired protein as described above. A wide variety of promoters may be utilized within the context of the present invention, including for example, promoters such as MoMLV LTR, RSV LTR, Friend MuLV LTR, adenoviral promoter (Ohno et al., Science 265:781-784, 1994), neomycin phosphotransferase promoter/enhancer, late parvovirus promoter (Koering et al., Hum. Gene Therap. 5:457-463, 1994), Herpes TK promoter, SV40 promoter, metallothionein IIa gene enhancer/promoter, cytomegalovirus immediate early promoter, and the cytomegalovirus immediate late promoter. Within particularly preferred embodiments of the invention, the promoter is a tissue-specific promoter (see e.g., WO 91/02805; EP 0,415,731; and WO 90/07936). Representative examples of suitable tissue specific promoters include neural specific enolase promoter, platelet derived growth factor beta promoter, bone morphogenic protein promoter, human alpha1-chimaerin promoter, synapsin I promoter and synapsin II promoter. In addition to the above-noted promoters, other viral-specific promoters (e.g., retroviral promoters (including those noted above, as well as others such as HIV promoters), hepatitis, herpes (e.g., EBV), and bacterial, fungal or parasitic (e.g., malarial) -specific promoters may be utilized in order to target a specific cell or tissue which is infected with a virus, bacteria, fungus or parasite.

Mammalian cells suitable for carrying out the present invention include, among others COS, CHO, SaOS, osteosarcomas, KS483, MG-63, primary osteoblasts, and human or mammalian bone marrow stroma. Mammalian expression vectors for use in carrying out the present invention will include a promoter capable of directing the transcription of a cloned gene or cDNA. Preferred promoters include viral promoters and cellular promoters. Bone specific promoters include the bone sialo-protein and the promoter for osteocalcin. Viral promoters include the cytomegalovirus immediate early promoter (Boshart et al., Cell 41:521-530, 1985), cytomegalovirus immediate late promoter, SV40 promoter (Subramani et al., Mol. Cell. Biol. 1:854-864, 1981), MMTV LTR, RSV LTR, metallothionein-1, adenovirus E1a. Cellular promoters include the mouse metallothionein-1 promoter (Palmiter et al., U.S. Patent No. 4,579,821), a

10

15

mouse V_K promoter (Bergman et al., *Proc. Natl. Acad. Sci. USA 81*:7041-7045, 1983; Grant et al., *Nucl. Acids Res. 15*:5496, 1987) and a mouse V_H promoter (Loh et al., *Cell 33*:85-93, 1983). The choice of promoter will depend, at least in part, upon the level of expression desired or the recipient cell line to be transfected.

Such expression vectors may also contain a set of RNA splice sites located downstream from the promoter and upstream from the DNA sequence encoding the peptide or protein of interest. Preferred RNA splice sites may be obtained from adenovirus and/or immunoglobulin genes. Also contained in the expression vectors is a polyadenylation signal located downstream of the coding sequence of interest. Suitable polyadenylation signals include the early or late polyadenylation signals from SV40 (Kaufman and Sharp, *ibid.*), the polyadenylation signal from the Adenovirus 5 E1B region and the human growth hormone gene terminator (DeNoto et al., *Nuc. Acids Res.* 9:3719-3730, 1981). The expression vectors may include a noncoding viral leader sequence, such as the Adenovirus 2 tripartite leader, located between the promoter and the RNA splice sites. Preferred vectors may also include enhancer sequences, such as the SV40 enhancer. Expression vectors may also include sequences encoding the adenovirus VA RNAs. Suitable expression vectors can be obtained from commercial sources (e.g., Stratagene, La Jolla, California).

into cultured mammalian cells by, for example, calcium phosphate-mediated transfection (Wigler et al., Cell 14:725, 1978; Corsaro and Pearson, Somatic Cell Genetics 7:603, 1981; Graham and Van der Eb, Virology 52:456, 1973), electroporation (Neumann et al., EMBO J. 1:841-845, 1982), or DEAE-dextran mediated transfection (Ausubel et al. (eds.), Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY, 1987). To identify cells that have stably integrated the cloned DNA, a selectable marker is generally introduced into the cells along with the gene or cDNA of interest. Preferred selectable markers for use in cultured mammalian cells include genes that confer resistance to drugs, such as neomycin, hygromycin, and methotrexate. The selectable marker may be an amplifiable selectable marker. Preferred amplifiable selectable markers are the DHFR gene and the neomycin resistance gene. Selectable markers are reviewed by Thilly (Mammalian Cell Technology, Butterworth Publishers, Stoneham, Massachusetts, which is incorporated herein by reference).

Mammalian cells containing a suitable vector are allowed to grow for a period of time, typically 1-2 days, to begin expressing the DNA sequence(s) of interest.

Drug selection is then applied to select for growth of cells that are expressing the selectable marker in a stable fashion. For cells that have been transfected with an

10

amplifiable, selectable marker the drug concentration may be increased in a stepwise manner to select for increased copy number of the cloned sequences, thereby increasing expression levels. Cells expressing the introduced sequences are selected and screened for production of the protein of interest in the desired form or at the desired level. Cells that satisfy these criteria can then be cloned and scaled up for production.

Protocols for the transfection of mammalian cells are well known to those of ordinary skill in the art. Representative methods include calcium phosphate mediated transfection, electroporation, lipofection, retroviral, adenoviral and protoplast fusion-mediated transfection (see Sambrook et al., supra). Naked vector constructs can also be taken up by muscular cells or other suitable cells subsequent to injection into the muscle of a mammal (or other animals).

Numerous insect host cells known in the art can also be useful within the present invention, in light of the subject specification. For example, the use of baculoviruses as vectors for expressing heterologous DNA sequences in insect cells has been reviewed by Atkinson et al. (*Pestic. Sci. 28*:215-224,1990).

Numerous plant host cells known in the art can also be useful within the present invention, in light of the subject specification. For example, the use of Agrobacterium rhizogenes as vectors for expressing genes in plant cells has been reviewed by Sinkar et al. (J. Biosci. (Bangalore) 11:47-58, 1987).

20 Within related aspects of the present invention, proteins of the present invention may be expressed in a transgenic animal whose germ cells and somatic cells contain a gene which encodes the desired protein and which is operably linked to a promoter effective for the expression of the gene. Alternatively, in a similar manner transgenic animals may be prepared that lack the desired gene (e.g., "knock-out" mice). Such transgenics may be prepared in a variety of non-human animals, including mice, rats, rabbits, sheep, dogs, goats and pigs (see Hammer et al., Nature 315:680-683, 1985, Palmiter et al., Science 222:809-814, 1983, Brinster et al., Proc. Natl. Acad. Sci. USA 82:4438-4442, 1985, Palmiter and Brinster, Cell 41:343-345, 1985, and U.S. Patent Nos. 5,175,383, 5,087,571, 4,736,866, 5,387,742, 5,347,075, 5,221,778, and 5,175,384). Briefly, an expression vector, including a nucleic acid molecule to be expressed together with appropriately positioned expression control sequences, is introduced into pronuclei of fertilized eggs, for example, by microinjection. Integration of the injected DNA is detected by blot analysis of DNA from tissue samples. It is preferred that the introduced DNA be incorporated into the germ line of the animal so that it is passed on to the animal's progeny. Tissue-specific expression may be 35 achieved through the use of a tissue-specific promoter, or through the use of an

20

25

inducible promoter, such as the metallothionein gene promoter (Palmiter et al., 1983, ibid), which allows regulated expression of the transgene.

Proteins can be isolated by, among other methods, culturing suitable host and vector systems to produce the recombinant translation products of the present invention. Supernatants from such cell lines, or protein inclusions or whole cells where the protein is not excreted into the supernatant, can then be treated by a variety of purification procedures in order to isolate the desired proteins. For example, the supernatant may be first concentrated using commercially available protein concentration filters, such as an Amicon or Millipore Pellicon ultrafiltration unit. Following concentration, the concentrate may be applied to a suitable purification matrix such as, for example, an anti-protein antibody bound to a suitable support. Alternatively, anion or cation exchange resins may be employed in order to purify the protein. As a further alternative, one or more reverse-phase high performance liquid chromatography (RP-HPLC) steps may be employed to further purify the protein. Other methods of isolating the proteins of the present invention are well known in the skill of the art.

A protein is deemed to be "isolated" within the context of the present invention if no other (undesired) protein is detected pursuant to SDS-PAGE analysis followed by Coomassie blue staining. Within other embodiments, the desired protein can be isolated such that no other (undesired) protein is detected pursuant to SDS-PAGE analysis followed by silver staining.

3. Nucleic Acid Molecules

Within other aspects of the invention, nucleic acid molecules are provided which are capable of inhibiting TGF-beta binding-protein binding to a member of the TGF-beta family. For example, within one embodiment antisense oligonucleotide molecules are provided which specifically inhibit expression of TGF-beta binding-protein nucleic acid sequences (see generally, Hirashima et al. in Molecular Biology of RNA: New Perspectives (M. Inouye and B. S. Dudock, eds., 1987 Academic Press, San Diego, p. 401); Oligonucleotides: Antisense Inhibitors of Gene Expression (J.S. Cohen, ed., 1989 MacMillan Press, London); Stein and Cheng, Science 261:1004-1012, 1993; WO 95/10607; U.S. Patent No. 5,359,051; WO 92/06693; and EP-A2-612844). Briefly, such molecules are constructed such that they are complementary to, and able to form Watson-Crick base pairs with, a region of transcribed TGF-beta binding-protein mRNA sequence. The resultant double-stranded

nucleic acid interferes with subsequent processing of the mRNA, thereby preventing protein synthesis (see Example 10).

Within other aspects of the invention, ribozymes are provided which are capable of inhibiting the TGF-beta binding-protein binding to a member of the TGFbeta family. As used herein, "ribozymes" are intended to include RNA molecules that contain anti-sense sequences for specific recognition, and an RNA-cleaving enzymatic activity. The catalytic strand cleaves a specific site in a target RNA at greater than stoichiometric concentration. A wide variety of ribozymes may be utilized within the context of the present invention, including for example, the hammerhead ribozyme (for example, as described by Forster and Symons, Cell 48:211-220, 1987; Haseloff and Gerlach, Nature 328:596-600, 1988; Walbot and Bruening, Nature 334:196, 1988; Haseloff and Gerlach, Nature 334:535, 1988); the hairpin ribozyme (for example, as described by Haseloff et al., U.S. Patent No. 5,254,678, issued October 19, 1993 and Hempel et al., European Patent Publication No. 0 360 257, published March 26, 1990); and Tetrahymena ribosomal RNA-based ribozymes (see Cech et al., U.S. Patent No. 4,987,071). Ribozymes of the present invention typically consist of RNA, but may also be composed of DNA, nucleic acid analogs (e.g., phosphorothioates), or chimerics thereof (e.g., DNA/RNA/RNA).

4. Labels

15

20

30

35

The gene product or any of the candidate molecules described above and below, may be labeled with a variety of compounds, including for example, fluorescent molecules, toxins, and radionuclides. Representative examples of fluorescent molecules include fluorescein, *Phycobili* proteins, such as phycoerythrin, rhodamine, Texas red and luciferase. Representative examples of toxins include ricin, abrin diphtheria toxin, cholera toxin, gelonin, pokeweed antiviral protein, tritin, *Shigella* toxin, and *Pseudomonas* exotoxin A. Representative examples of radionuclides include Cu-64, Ga-67, Ga-68, Zr-89, Ru-97, Tc-99m, Rh-105, Pd-109, In-111, I-123, I-125, I-131, Re-186, Re-188, Au-198, Au-199, Pb-203, At-211, Pb-212 and Bi-212. In addition, the antibodies described above may also be labeled or conjugated to one partner of a ligand binding pair. Representative examples include avidin-biotin, and riboflavin-riboflavin binding protein.

Methods for conjugating or labeling the molecules described herein with the representative labels set forth above may be readily accomplished by one of ordinary skill in the art (see Trichothecene Antibody Conjugate, U.S. Patent No. 4,744,981; Antibody Conjugate, U.S. Patent No. 5,106,951; Fluorogenic Materials and

15

20

25

Labeling Techniques, U.S. Patent No. 4,018,884; Metal Radionuclide Labeled Proteins for Diagnosis and Therapy, U.S. Patent No. 4,897,255; and Metal Radionuclide Chelating Compounds for Improved Chelation Kinetics, U.S. Patent No. 4,988,496; see also Inman, Methods In Enzymology, Vol. 34, Affinity Techniques, Enzyme Purification: Part B, Jakoby and Wilchek (eds.), Academic Press, New York, p. 30, 1974; see also Wilchek and Bayer, "The Avidin-Biotin Complex in Bioanalytical Applications," Anal. Biochem. 171:1-32, 1988).

PHARMACEUTICAL COMPOSITIONS

As noted above, the present invention also provides a variety of pharmaceutical compositions, comprising one of the above-described molecules which inhibits the TGF-beta binding-protein binding to a member of the TGF-beta family along with a pharmaceutically or physiologically acceptable carrier, excipients or diluents. Generally, such carriers should be nontoxic to recipients at the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining the therapeutic agent with buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or dextrins, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with nonspecific serum albumin are exemplary appropriate diluents.

In addition, the pharmaceutical compositions of the present invention may be prepared for administration by a variety of different routes. In addition, pharmaceutical compositions of the present invention may be placed within containers, along with packaging material which provides instructions regarding the use of such pharmaceutical compositions. Generally, such instructions will include a tangible expression describing the reagent concentration, as well as within certain embodiments, relative amounts of excipient ingredients or diluents (e.g., water, saline or PBS) which may be necessary to reconstitute the pharmaceutical composition.

METHODS OF TREATMENT

The present invention also provides methods for increasing the mineral content and mineral density of bone. Briefly, numerous conditions result in the loss of bone mineral content, including for example, disease, genetic predisposition, accidents which result in the lack of use of bone (e.g., due to fracture), therapeutics which effect bone resorption, or which kill bone forming cells and normal aging. Through use of the molecules described herein which inhibit the TGF-beta binding-protein binding to a

TGF-beta family member such conditions may be treated or prevented. As utilized herein, it should be understood that bone mineral content has been increased, if bone mineral content has been increased in a statistically significant manner (e.g., greater than one-half standard deviation), at a selected site.

A wide variety of conditions which result in loss of bone mineral content may be treated with the molecules described herein. Patients with such conditions may be identified through clinical diagnosis utilizing well known techniques (see, e.g., Harrison's Principles of Internal Medicine, McGraw-Hill, Inc.). Representative examples of diseases that may be treated included dysplasias, wherein there is abnormal growth or development of bone. Representative examples of such conditions include achondroplasia, cleidocranial dysostosis, enchondromatosis, fibrous dysplasia, Gaucher's, hypophosphatemic rickets, Marfan's, multiple hereditary exotoses, neurofibromatosis, osteogenesis imperfecta, osteopetrosis, osteopoikilosis, sclerotic lesions, fractures, periodontal disease, pseudoarthrosis and pyogenic osteomyelitis.

Other conditions which may be treated or prevented include a wide variety of causes of osteopenia (i.e., a condition that causes greater than one standard deviation of bone mineral content or density below peak skeletal mineral content at youth). Representative examples of such conditions include anemic states, conditions caused steroids, conditions caused by heparin, bone marrow disorders, scurvy, malnutrition, calcium deficiency, idiopathic osteoporosis, congenital osteopenia or osteoporosis, alcoholism, chronic liver disease, senility, postmenopausal state, oligomenorrhea, amenorrhea, pregnancy, diabetes mellitus, hyperthyroidism, Cushing's disease, acromegaly, hypogonadism, immobilization or disuse, reflex sympathetic dystrophy syndrome, transient regional osteoporosis and osteomalacia.

Within one aspect of the present invention, bone mineral content or density may be increased by administering to a warm-blooded animal a therapeutically effective amount of a molecule which inhibits the TGF-beta binding-protein binding to a TGF-beta family member. Examples of warm-blooded animals that may be treated include both vertebrates and mammals, including for example horses, cows, pigs, sheep, dogs, cats, rats and mice. Representative examples of therapeutic molecules include ribozymes, ribozyme genes, antisense oligonucleotides and antibodies (e.g, humanized antibodies).

Within other aspects of the present invention, methods are provided for increasing bone density, comprising the step of introducing into cells which home to bone a vector which directs the expression of a molecule which inhibits the TGF-beta binding-protein binding to a member of the TGF-beta family, and administering the

35

5

10

15

vector containing cells to a warm-blooded animal. Briefly, cells which home to bone may be obtained directly from the bone of patients (e.g., cells obtained from the bone marrow such as CD34+, osteoblasts, osteocytes, and the like), from peripheral blood, or from cultures.

A vector which directs the expression of a molecule that inhibits the 5 TGF-beta binding-protein binding to a member of the TGF-beta family is introduced into the cells. Representative examples of suitable vectors include viral vectors such as herpes viral vectors (e.g., U.S. Patent No. 5,288,641), adenoviral vectors (e.g., WO 94/26914, WO 93/9191; Kolls et al., PNAS 91(1):215-219, 1994; Kass-Eisler et al., PNAS 90(24):11498-502, 1993; Guzman et al., Circulation 88(6):2838-48, 1993; Guzman et al., Cir. Res. 73(6):1202-1207, 1993; Zabner et al., Cell 75(2):207-216, 1993; Li et al., Hum Gene Ther. 4(4):403-409, 1993; Caillaud et al., Eur. J. Neurosci. 5(10:1287-1291, 1993; Vincent et al., Nat. Genet. 5(2):130-134, 1993; Jaffe et al., Nat. Genet. 1(5):372-378, 1992; and Levrero et al., Gene 101(2):195-202, 1991), adenoassociated viral vectors (WO 95/13365; Flotte et al., PNAS 90(22):10613-10617, 1993), baculovirus vectors, parvovirus vectors (Koering et al., Hum. Gene Therap. 5:457-463, 1994), pox virus vectors (Panicali and Paoletti, PNAS 79:4927-4931, 1982; and Ozaki et al., Biochem. Biophys. Res. Comm. 193(2):653-660, 1993), and retroviruses (e.g., EP 0,415,731; WO 90/07936; WO 91/0285, WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent No. 5,219,740; WO 93/11230; WO 93/10218). Viral 20 vectors may likewise be constructed which contain a mixture of different elements (e.g., promoters, envelope sequences and the like) from different viruses, or non-viral sources. Within various embodiments, either the viral vector itself, or a viral particle which contains the viral vector may be utilized in the methods and compositions 25 described below.

Within other embodiments of the invention, nucleic acid molecules which encode a molecule which inhibits the TGF-beta binding-protein binding to a member of the TGF-beta family themselves may be administered by a variety of techniques, including, for example, administration of asialoosomucoid (ASOR) conjugated with poly-L-lysine DNA complexes (Cristano et al., *PNAS* 92122-92126, 1993), DNA linked to killed adenovirus (Curiel et al., *Hum. Gene Ther.* 3(2):147-154, 1992), cytofectin-mediated introduction (DMRIE-DOPE, Vical, California), direct DNA injection (Acsadi et al., *Nature* 352:815-818, 1991); DNA ligand (Wu et al., *J. of Biol. Chem.* 264:16985-16987, 1989); lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7417, 1989); liposomes (Pickering et al., *Circ.* 89(1):13-21, 1994; and Wang et al., *PNAS* 84:7851-7855, 1987); microprojectile bombardment (Williams

30

15

20

et al., PNAS 88:2726-2730, 1991); and direct delivery of nucleic acids which encode the protein itself either alone (Vile and Hart, Cancer Res. 53: 3860-3864, 1993), or utilizing PEG-nucleic acid complexes.

Representative examples of molecules which may be expressed by the vectors of present invention include ribozymes and antisense molecules, each of which are discussed in more detail above.

Determination of increased bone mineral content may be determined directly through the use of X-rays (e.g., Dual Energy X-ray Absorptometry or "DEXA"), or by inference through bone turnover markers (osteoblast specific alkaline phosphatase, osteocalcin, type 1 procollagen C' propeptide (PICP), and total alkaline phosphatase; see Comier, C., Curr. Opin. in Rheu. 7:243, 1995), or markers of bone resorption (pyridinoline, deoxypryridinoline, N-telopeptide, urinary hydroxyproline, plasma tartrate-resistant acid phosphatases and galactosyl hydroxylysine; see Comier, supra). The amount of bone mass may also be calculated from body weights, or utilizing other methods (see Guinness-Hey, Metab. Bone Dis. and Rel. Res. 5:177-181, 1984).

As will be evident to one of skill in the art, the amount and frequency of administration will depend, of course, on such factors as the nature and severity of the indication being treated, the desired response, the condition of the patient, and so forth. Typically, the compositions may be administered by a variety of techniques, as noted above.

The following examples are offered by way of illustration, and not by way of limitation.

20

25

30

EXAMPLES EXAMPLE 1

SCLEROSTEOSIS MAPS TO THE LONG ARM OF HUMAN CHROMOSOME 17

Genetic mapping of the defect responsible for sclerosteosis in humans localized the gene responsible for this disorder to the region of human chromosome 17 that encodes a novel TGF-beta binding-protein family member. In sclerosteosis, skeletal bone displays a substantial increase in mineral density relative to that of unafflicted individuals. Bone in the head displays overgrowth as well. Sclerosteosis patients are generally healthy although they may exhibit variable degrees of syndactyly at birth and variable degrees of cranial compression and nerve compression in the skull.

Linkage analysis of the gene defect associated with sclerosteosis was conducted by applying the homozygosity mapping method to DNA samples collected from 24 South African Afrikaaner families in which the disease occurred. (Sheffield et al., 1994, *Human Molecular Genetics 3*:1331-1335. "Identification of a Bardet-Biedl syndrome locus on chromosome 3 and evaluation of an efficient approach to homozygosity mapping"). The Afrikaaner population of South Africa is genetically homogeneous; the population is descended from a small number of founders who colonized the area several centuries ago, and it has been isolated by geographic and social barriers since the founding. Sclerosteosis is rare everywhere in the world outside the Afrikaaner community, which suggests that a mutation in the gene was present in the founding population and has since increased in numbers along with the increase in the population. The use of homozygosity mapping is based on the assumption that DNA mapping markers adjacent to a recessive mutation are likely to be homozygous in affected individuals from consanguineous families and isolated populations.

A set of 371 microsatellite markers (Research Genetics, Set 6) from the autosomal chromosomes was selected to type pools of DNA from sclerosteosis patient samples. The DNA samples for this analysis came from 29 sclerosteosis patients in 24 families, 59 unaffected family members and a set of unrelated control individuals from the same population. The pools consisted of 4-6 individuals, either affected individuals, affected individuals from consanguineous families, parents and unaffected siblings, or unrelated controls. In the pools of unrelated individuals and in most of the pools with affected individuals or family members analysis of the markers showed several allele sizes for each marker. One marker, D17S1299, showed an indication of homozygosity: one band in several of the pools of affected individuals.

All 24 sclerosteosis families were typed with a total of 19 markers in the region of D17S1299 (at 17q12-q21). Affected individuals from every family were shown to be homozygous in this region, and 25 of the 29 individuals were homozygous for a core haplotype; they each had the same alleles between D17S1787 and D17S930. The other four individuals had one chromosome which matched this haplotype and a second which did not. In sum, the data compellingly suggested that this 3 megabase region contained the sclerosteosis mutation. Sequence analysis of most of the exons in this 3 megabase region identified a nonsense mutation in the novel TGF-beta binding-protein coding sequence (C>T mutation at position 117 of Sequence ID No. 1 results in a stop codon). This mutation was shown to be unique to sclerosteosis patients and carriers of Afrikaaner descent. The identity of the gene was further confirmed by identifying a mutation in its intron (A>T mutation at position +3 of the intron) which results in improper mRNA processing in a single, unrelated patient with diagnosed sclerosteosis.

15

20

25

10

EXAMPLE 2

TISSUE-SPECIFICITY OF TGF-BETA BINDING-PROTEIN GENE EXPRESSION
A. Human Beer Gene Expression by RT-PCR:

First-strand cDNA was prepared from the following total RNA samples using a commercially available kit ("Superscript Preamplification System for First-Strand cDNA Synthesis", Life Technologies, Rockville, MD): human brain, human liver, human spleen, human thymus, human placenta, human skeletal muscle, human thyroid, human pituitary, human osteoblast (NHOst from Clonetics Corp., San Diego, CA), human osteosarcoma cell line (Saos-2, ATCC# HTB-85), human bone, human bone marrow, human cartilage, vervet monkey bone, saccharomyces cerevisiae, and human peripheral blood monocytes. All RNA samples were purchased from a commercial source (Clontech, Palo Alto, CA), except the following which were prepared in-house: human osteoblast, human osteosarcoma cell line, human bone, human cartilage and vervet monkey bone. These in-house RNA samples were prepared using a commercially available kit ("TRI Reagent", Molecular Research Center, Inc., Cincinnati, OH).

PCR was performed on these samples, and additionally on a human genomic sample as a control. The sense Beer oligonucleotide primer had the sequence 5'-CCGGAGCTGGAGAACAACAAG-3' (SEQ ID NO:19). The antisense Beer oligonucleotide primer had the sequence 5'-GCACTGGCCGGAGCACACC-3' (SEQ

WO 00/32773 PCT/US99/27990

56

ID NO:20). In addition, PCR was performed using primers for the human beta-actin gene, as a control. The sense beta-actin oligonucleotide primer had the sequence 5'-AGGCCAACCGCGAGAAGATGA CC -3' (SEQ ID NO:21). The antisense beta-actin oligonucleotide primer had the sequence 5'-GAAGT CCAGGGCGACGTAGCA-3' (SEQ ID NO:22). PCR was performed using standard conditions in 25 ul reactions, with an annealing temperature of 61 degrees Celsius. Thirty-two cycles of PCR were performed with the Beer primers and twenty-four cycles were performed with the beta-actin primers.

Following amplification, 12 ul from each reaction were analyzed by agarose gel electrophoresis and ethidium bromide staining. See Figure 2A.

B. RNA In-situ Hybridization of Mouse Embryo Sections:

The full length mouse *Beer* cDNA (Sequence ID No. 11) was cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) in the antisense and sense direction using the manufacturer's protocol. ³⁵S-alpha-GTP-labeled cRNA sense and antisense transcripts were synthesized using in-vitro transcription reagents supplied by Ambion, Inc (Austin, TX). In-situ hybridization was performed according to the protocols of Lyons et al. (*J. Cell Biol. 111*:2427-2436, 1990).

The mouse *Beer* cRNA probe detected a specific message expressed in the neural tube, limb buds, blood vessels and ossifying cartilages of developing mouse embryos. Panel A in Figure 3 shows expression in the apical ectodermal ridge (aer) of the limb (1) bud, blood vessels (bv) and the neural tube (nt). Panel B shows expression in the 4th ventricle of the brain (4). Panel C shows expression in the mandible (ma) cervical vertebrae (cv), occipital bone (oc), palate (pa) and a blood vessel (bv). Panel D shows expression in the ribs (r) and a heart valve (va). Panel A is a transverse section of 10.5 dpc embryo. Panel B is a sagittal section of 12.5 dpc embryo and panels C and D are sagittal sections of 15.5 dpc embryos. ba=branchial arch, h=heart, te=telencephalon (forebrain), b=brain, f=frontonasal mass, g=gut, h=heart, j=jaw, li=liver, lu=lung, ot=otic vesicle, ao=, sc=spinal cord, skm=skeletal muscle, ns=nasal sinus, th=thymus , to=tongue, f1=forelimb, di=diaphragm

EXAMPLE 3

35 EXPRESSION AND PURIFICATION OF RECOMBINANT BEER PROTEIN

A. Expression in COS-1 Cells:

20

The DNA sequence encoding the full length human Beer protein was amplified using the following PCR oligonucleotide primers: The 5' oligonucleotide primer had the sequence 5'-AAGCTTGGTACCATGCAGCTCCCAC-3' (SEQ ID NO:23) and contained a HindIII restriction enzyme site (in bold) followed by 19 nucleotides of the Beer gene starting 6 base pairs prior to the presumed amino terminal start codon (ATG). The 3' oligonucleotide primer had the sequence 5'-AAGCTTCTACTTGTCATCGTCGTCCT_TGTAGTCGTAGGCGTTCTCCAGCT-3' (SEQ ID NO:24) and contained a HindIII restriction enzyme site (in bold) followed by a reverse complement stop codon (CTA) followed by the reverse complement of the FLAG epitope (underlined, Sigma-Aldrich Co., St. Louis, MO) flanked by the reverse complement of nucleotides coding for the carboxy terminal 5 amino acids of the Beer. The PCR product was TA cloned ("Original TA Cloning Kit", Invitrogen, Carlsbad, CA) and individual clones were screened by DNA sequencing. A sequence-verified clone was then digested by HindIII and purified on a 1.5% agarose gel using a commercially available reagents ("QIAquick Gel Extraction Kit", Qiagen Inc., Valencia, CA). This fragment was then ligated to HindIII digested, phosphatase-treated pcDNA3.1 (Invitrogen, Carlsbad, CA) plasmid with T4 DNA ligase. DH10B E. coli were transformed and plated on LB, $100~\mu\text{g/ml}$ ampicillin plates. Colonies bearing the desired recombinant in the proper orientation were identified by a PCR-based screen, using a 5' primer corresponding to the T7 promoter/priming site in pcDNA3.1 and a 3' primer with the sequence 5'- GCACTGGCCGGAGCACACC-3' (SEQ ID NO:25) that corresponds to the reverse complement of internal BEER sequence. The sequence of the cloned fragment was confirmed by DNA sequencing.

the expression plasmid pcDNA-Beer-Flag was transfected using a commercially available kit following protocols supplied by the manufacturer ("DEAE-Dextran Transfection Kit", Sigma Chemical Co., St. Louis, MO). The final media following transfection was DMEM (Life Technologies, Rockville, MD) containing 0.1% Fetal Bovine Serum. After 4 days in culture, the media was removed. Expression of recombinant BEER was analyzed by SDS-PAGE and Western Blot using anti-FLAG M2 monoclonal antibody (Sigma-Aldrich Co., St. Louis, MO). Purification of recombinant BEER protein was performed using an anti-FLAG M2 affinity column ("Mammalian Transient Expression System", Sigma-Aldrich Co., St. Louis, MO). The column profile was analyzed via SDS-PAGE and Western Blot using anti-FLAG M2 monoclonal antibody.

B. Expression in SF9 insect cells:

The human Beer gene sequence was amplified using PCR with standard conditions and the following primers:

Sense primer: 5'-GTCGTCGGATCCATGGGGTGGCAGGCGTTCAAGAATGAT-3' (SEQ ID NO:26)

Antisense primer: 5'-GTCGTCAAGCTTCTACTTGTCATCGTCCTTGTAGTCGTA GGCGTTCTCCAGCTCGGC-3' (SEQ ID NO:27)

The resulting cDNA contained the coding region of Beer with two modifications. The N-terminal secretion signal was removed and a FLAG epitope tag (Sigma) was fused in frame to the C-terminal end of the insert. BamHl and HindIII cloning sites were added and the gene was subcloned into pMelBac vector (Invitrogen) for transfer into a baculoviral expression vector using standard methods.

Recombinant baculoviruses expressing Beer protein were made using the Bac-N-Blue transfection kit (Invitrogen) and purified according to the manufacturers instructions.

SF9 cells (Invitrogen) were maintained in TNM_FH media (Invitrogen) containing 10% fetal calf serum. For protein expression, SF9 cultures in spinner flasks were infected at an MOI of greater than 10. Samples of the media and cells were taken daily for five days, and Beer expression monitored by western blot using an anti-FLAG M2 monoclonal antibody (Sigma) or an anti-Beer rabbit polyclonal antiserum.

After five days the baculovirus-infected SF9 cells were harvested by centrifugation and cell associated protein was extracted from the cell pellet using a high salt extraction buffer (1.5 M NaCl, 50 mM Tris pH 7.5). The extract (20 ml per 300 ml culture) was clarified by centrifugation, dialyzed three times against four liters of Tris buffered saline (150 mM NaCl, 50 mM Tris pH 7.5), and clarified by centrifugation again. This high salt fraction was applied to Hitrap Heparin (Pharmacia; 5 ml bed volume), washed extensively with HEPES buffered saline (25 mM HEPES 7.5, 150 mM Nacl) and bound proteins were eluted with a gradient from 150 mM NaCl to 1200 mM NaCl. Beer elution was observed at aproximately 800 mM NaCl. Beer containing fractions were supplemented to 10% glycerol and 1 mM DTT and frozen at -80 degrees C.

EXAMPLE 4

PREPARATION AND TESTING OF POLYCLONAL ANTIBODIES TO BEER, GREMLIN, AND DAN

15

A. Preparation of antigen:

The DNA sequences of Human Beer, Human Gremlin, and Human Dan were amplified using standard PCR methods with the following oligonucleotide primers:

5 H. Beer

Sense: 5'-GACTTGGATCCCAGGGGTGGCAGGCGTTC- 3' (SEQ ID NO:28)
Antisense 5'-AGCATAAGCTTCTAGTAGGCGTTCTCCAG- 3' (SEQ ID NO:29)
H. Gremlin

Sense: 5' -GACTTGGATCCGAAGGGAAAAAGAAAGGGG- 3' (SEQ ID NO:30)

10 Antisense: 5' -AGCATAAGCTTTTAATCCAAATCGATGGA- 3' (SEQ ID NO:31)

H. Dan

Sense: 5' -ACTACGAGCTCGGCCCCACCACCATCAACAAG- 3' (SEQ ID NO:32)
Antisense: 5' -ACTTAGAAGCTTTCAGTCCTCAGCCCCCTCTTCC-3' (SEQ ID NO:33)

In each case the listed primers amplified the entire coding region minus the secretion signal sequence. These include restriction sites for subcloning into the bacterial expression vector pQE-30 (Qiagen Inc., Valencia, CA) at sites BamHI/HindIII for Beer and Gremlin, and sites SacI/HindIII for Dan. pQE30 contains a coding sequence for a 6x His tag at the 5' end of the cloning region. The completed constructs were transformed into *E. coli* strain M-15/pRep (Qiagen Inc) and individual clones verified by sequencing. Protein expression in M-15/pRep and purification (6xHis affinity tag binding to Ni-NTA coupled to Sepharose) were performed as described by the manufacturer (Qiagen, The QIAexpressionist).

The *E. coli*-derived Beer protein was recovered in significant quantity using solubilization in 6M guanidine and dialyzed to 2-4M to prevent precipitation during storage. Gremlin and Dan protein were recovered in higher quantity with solubilization in 6M guanidine and a post purification guanidine concentration of 0.5M.

B. Production and testing of polyclonal antibodies:

Polyclonal antibodies to each of the three antigens were produced in rabbit and in chicken hosts using standard protocols (R & R Antibody, Stanwood, WA; standard protocol for rabbit immunization and antisera recovery; Short Protocols in Molecular Biology. 2nd edition. 1992. 11.37-11.41. Contributors Helen M. Cooper and Yvonne Paterson; chicken antisera was generated with Strategic Biosolutions, Ramona. CA).

Rabbit antisera and chicken egg Igy fraction were screened for activity

via Western blot. Each of the three antigens was separated by PAGE and transferred to 0.45um nitrocellulose (Novex, San Diego, CA). The membrane was cut into strips with each strip containing approximately 75 ng of antigen. The strips were blocked in 3% Blotting Grade Block (Bio-Rad Laboratories, Hercules, CA) and washed 3 times in 1X Tris buffer saline (TBS) /0.02% TWEEN buffer. The primary antibody (preimmunization bleeds, rabbit antisera or chicken egg IgY in dilutions ranging from 1:100 to 1:10,000 in blocking buffer) was incubated with the strips for one hour with gentle rocking. A second series of three washes 1X TBS/0.02%TWEEN was followed by an one hour incubation with the secondary antibody (peroxidase conjugated donkey anti-rabbit, Amersham Life Science, Piscataway, NJ; or peroxidase conjugated donkey anti-chicken, Jackson ImmunoResearch, West Grove, PA). A final cycle of 3X washes of 1X TBS/0.02%TWEEN was performed and the strips were developed with Lumi-Light Western Blotting Substrate (Roche Molecular Biochemicals, Mannheim, Germany).

15

20

10

C. Antibody cross-reactivity test:

Following the protocol described in the previous section, nitrocellulose strips of Beer, Gremlin or Dan were incubated with dilutions (1:5000 and 1:10,000) of their respective rabbit antisera or chicken egg IgY as well as to antisera or chicken egg Igy (dilutions 1:1000 and 1:5000) made to the remaining two antigens. The increased levels of nonmatching antibodies was performed to detect low affinity binding by those antibodies that may be seen only at increased concentration. The protocol and duration of development is the same for all three binding events using the protocol described above. There was no antigen cross-reactivity observed for any of the antigens tested.

25

EXAMPLE 5

INTERACTION OF BEER WITH TGF-BETA SUPER-FAMILY PROTEINS

The interaction of Beer with proteins from different phylogenetic arms of the TGF-β superfamily were studied using immunoprecipitation methods. Purified TGFβ-1, TGFβ-2, TGFβ-3, BMP-4, BMP-5, BMP-6 and GDNF were obtained from commercial sources (R&D systems; Minneapolis, MN). A representative protocol is as follows. Partially purified Beer was dialyzed into HEPES buffered saline (25 mM HEPES 7.5, 150 mM NaCl). Immunoprecipitations were done in 300 ul of IP buffer (150 mM NaCl, 25 mM Tris pH 7.5, 1mM EDTA, 1.4 mM β-mercaptoethanol, 0.5 % triton X 100, and 10% glycerol). 30 ng recombinant human BMP-5 protein (R&D

systems) was applied to 15 ul of FLAG affinity matrix (Sigma; St Louis MO)) in the presence and absence of 500 ng FLAG epitope-tagged Beer. The proteins were incubated for 4 hours @ 4°Cand then the affinity matrix-associated proteins were washed 5 times in IP buffer (1 ml per wash). The bound proteins were eluted from the affinity matrix in 60 microliters of 1X SDS PAGE sample buffer. The proteins were resolved by SDS PAGE and Beer associated BMP-5 was detected by western blot using anti-BMP-5 antiserum (Research Diagnostics, Inc) (see Figure 5).

BEER Ligand Binding Assay:

FLAG-Beer protein (20 ng) is added to 100 ul PBS/0.2% BSA and adsorbed into each well of 96 well microtiter plate previously coated with anti-FLAG monoclonal antibody (Sigma; St Louis MO) and blocked with 10% BSA in PBS. This is conducted at room temperature for 60 minutes. This protein solution is removed and the wells are washed to remove unbound protein. BMP-5 is added to each well in concentrations ranging from 10 pM to 500 nM in PBS/0.2% BSA and incubated for 2 hours at room temperature. The binding solution is removed and the plate washed with three times with 200ul volumes of PBS/0.2% BSA. BMP-5 levels are then detected using BMP-5 anti-serum via ELISA (F.M. Ausubel et al (1998) Current Protocols in Mol Biol. Vol 2 11.2.1-11.2.22). Specific binding is calculated by subtracting non-specific binding from total binding and analyzed by the LIGAND program (Munson and Podbard, Anal. Biochem., 107, p220-239, (1980).

In a variation of this method, Beer is engineered and expressed as a human Fc fusion protein. Likewise the ligand BMP is engineered and expressed as mouse Fc fusion. These proteins are incubated together and the assay conducted as described by Mellor et al using homogeneous time resolved fluorescence detection (G.W. Mellor et al., *J of Biomol Screening*, 3(2) 91-99, 1998).

EXAMPLE 6

30

35

10

20

25

SCREENING ASSAY FOR INHIBITION OF TGF-BETA BINDING-PROTEIN BINDING TO TGF-BETA FAMILY MEMBERS

The assay described above is replicated with two exceptions. First, BMP concentration is held fixed at the Kd determined previously. Second, a collection of antagonist candidates is added at a fixed concentration (20 uM in the case of the small organic molecule collections and 1 uM in antibody studies). These candidate molecules (antagonists) of TGF-beta binding-protein binding include organic

compounds derived from commercial or internal collections representing diverse chemical structures. These compounds are prepared as stock solutions in DMSO and are added to assay wells at ≤ 1% of final volume under the standard assay conditions. These are incubated for 2 hours at room temperature with the BMP and Beer, the solution removed and the bound BMP is quantitated as described. Agents that inhibit 40% of the BMP binding observed in the absence of compound or antibody are considered antagonists of this interaction. These are further evaluated as potential inhibitors based on titration studies to determine their inhibition constants and their influence on TGF-beta binding-protein binding affinity. Comparable specificity control assays may also be conducted to establish the selectivity profile for the identified antagonist through studies using assays dependent on the BMP ligand action (e.g. BMP/BMP receptor competition study).

15

20

25

30

EXAMPLE 7

INHIBITION OF TGF-BETA BINDING-PROTEIN LOCALIZATION TO BONE MATRIX

Evaluation of inhibition of localization to bone matrix (hydroxyapatite) is conducted using modifications to the method of Nicolas (Nicolas, V. Calcif Tissue Int 57:206, 1995). Briefly, ¹²⁵I-labelled TGF-beta binding-protein is prepared as described by Nicolas (supra). Hydroxyapatite is added to each well of a 96 well microtiter plate equipped with a polypropylene filtration membrane (Polyfiltroninc, Weymouth MA). TGF-beta binding-protein is added to 0.2% albumin in PBS buffer. The wells containing matrix are washed 3 times with this buffer. Adsorbed TGF-beta binding-protein is eluted using 0.3M NaOH and quantitated.

Inhibitor identification is conducted via incubation of TGF-beta binding-protein with test molecules and applying the mixture to the matrix as described above. The matrix is washed 3 times with 0.2% albumin in PBS buffer. Adsorbed TGF-beta binding-protein is eluted using 0.3 M NaOH and quantitated. Agents that inhibit 40% of the TGF-beta binding-protein binding observed in the absence of compound or antibody are considered bone localization inhibitors. These inhibitors are further characterized through dose response studies to determine their inhibition constants and their influence on TGF-beta binding-protein binding affinity.

EXAMPLE 8

CONSTRUCTION OF TGF-BETA BINDING-PROTEIN MUTANT

Α. Mutagenesis:

A full-length TGF-beta binding-protein cDNA in pBluescript SK serves as a template for mutagenesis. Briefly, appropriate primers (see the discussion provided above) are utilized to generate the DNA fragment by polymerase chain reaction using Vent DNA polymerase (New England Biolabs, Beverly, MA). polymerase chain reaction is run for 23 cycles in buffers provided by the manufacturer using a 57°C annealing temperature. The product is then exposed to two restriction enzymes and after isolation using agarose gel electrophoresis, ligated back into pRBP4-503 from which the matching sequence has been removed by enzymatic digestion. Integrity of the mutant is verified by DNA sequencing.

Mammalian Cell Expression and Isolation of Mutant TGF-beta binding-protein: B.

The mutant TGF-beta binding-protein cDNAs are transferred into the 15 pcDNA3.1 mammalian expression vector described in EXAMPLE 3. After verifying the sequence, the resultant constructs are transfected into COS-1 cells, and secreted protein is purified as described in EXAMPLE 3.

20

EXAMPLE 9

ANIMAL MODELS -I

GENERATION OF TRANSGENIC MICE OVEREXPRESSING THE BEER GENE

The ~200 kilobase (kb) BAC clone 15G5, isolated from the CITB mouse genomic DNA library (distributed by Research Genetics, Huntsville, AL) was used to 25 determine the complete sequence of the mouse Beer gene and its 5' and 3' flanking regions. A 41 kb Sall fragment, containing the entire gene body, plus ~17 kb of 5' flanking and ~20 kb of 3' flanking sequence was sub-cloned into the BamHI site of the SuperCosI cosmid vector (Stratagene, La Jolla, CA) and propagated in the E. coli strain DH10B. From this cosmid construct, a 35 kb Mlul - Avill restriction fragment (Sequence No. 6), including the entire mouse Beer gene, as well as 17 kb and 14 kb of 5' and 3' flanking sequence, respectively, was then gel purified, using conventional means, and used for microinjection of mouse zygotes (DNX Transgenics; US Patent No. 4,873,191). Founder animals in which the cloned DNA fragment was integrated randomly into the genome were obtained at a frequency of 5-30% of live-born pups. The presence of the transgene was ascertained by performing Southern blot analysis of

genomic DNA extracted from a small amount of mouse tissue, such as the tip of a tail. DNA was extracted using the following protocol: tissue was digested overnight at 55° C in a lysis buffer containing 200 mM NaCl, 100 mM Tris pH8.5, 5 mM EDTA, 0.2% SDS and 0.5 mg/ml Proteinase K. The following day, the DNA was extracted once with phenol/chloroform (50:50), once with chloroform/isoamylalcohol (24:1) and precipitated with ethanol. Upon resuspension in TE (10mM Tris pH7.5, 1 mM EDTA) 8-10 ug of each DNA sample were digested with a restriction endonuclease, such as EcoRI, subjected to gel electrophoresis and transferred to a charged nylon membrane, such as HyBondN+ (Amersham, Arlington Heights, IL). The resulting filter was then hybridized with a radioactively labelled fragment of DNA deriving from the mouse Beer gene locus, and able to recognize both a fragment from the endogenous gene locus and a fragment of a different size deriving from the transgene. Founder animals were bred to normal non-transgenic mice to generate sufficient numbers of transgenic and non-transgenic progeny in which to determine the effects of Beer gene overexpression. For these studies, animals at various ages (for example, 1 day, 3 weeks, 6 weeks, 4 months) are subjected to a number of different assays designed to ascertain gross skeletal formation, bone mineral density, bone mineral content, osteoclast and osteoblast activity, extent of endochondral ossification, cartilage formation, etc. The transcriptional activity from the transgene may be determined by extracting RNA from various tissues, and using an RT-PCR assay which takes advantage of single nucleotide polymorphisms between the mouse strain from which the transgene is derived (129Sv/J) and the strain of mice used for DNA microinjection [(C57BL5/J x SJL/J)F2].

<u>ANIMAL MODELS - II</u>

DISRUPTION OF THE MOUSE BEER GENE BY HOMOLOGOUS RECOMBINATION

Homologous recombination in embryonic stem (ES) cells can be used to inactivate the endogenous mouse Beer gene and subsequently generate animals carrying the loss-of-function mutation. A reporter gene, such as the $E.\ coli\ \beta$ -galactosidase gene, was engineered into the targeting vector so that its expression is controlled by the endogenous Beer gene's promoter and translational initiation signal. In this way, the spatial and temporal patterns of Beer gene expression can be determined in animals carrying a targeted allele.

The targeting vector was constructed by first cloning the drug-selectable phosphoglycerate kinase (PGK) promoter driven neomycin-resistance gene (neo) cassette from pGT-N29 (New England Biolabs, Beverly, MA) into the cloning vector pSP72 (Promega, Madson, WI). PCR was used to flank the PGKneo cassette with

20

25

30

bacteriophage P1 loxP sites, which are recognition sites for the P1 Cre recombinase (Hoess et al., PNAS USA, 79:3398, 1982). This allows subsequent removal of the neoresistance marker in targeted ES cells or ES cell-derived animals (US Patent 4,959,317). The PCR primers were comprised of the 34 nucleotide (ntd) loxP sequence, 15-25 ntd complementary to the 5' and 3' ends of the PGKneo cassette, as well as restriction enzyme recognition sites (BamHI in the sense primer and EcoRI in the anti-sense primer) for cloning into pSP72. The sequence of the sense primer was 5'-

AATCTGGATCCATAACTTCGTATAGCATACATTATACGAAGTTATCTGCAG

GATTCGAGGGCCCCT-3' (SEQ ID NO:34); sequence of the anti-sense primer was
5'-AATCTGAATTCCACCGGTGTTAATTAAATAACTTCGT
ATAATGTATGCTATACGAAGTTATAGATCTAGAG TCAGCTTCTGA-3' (SEQ ID NO:35).

The next step was to clone a 3.6 kb Xhol-HindIII fragment, containing the E. coli β -galactosidase gene and SV40 polyadenylation signal from pSV β 15 (Clontech, Palo Alto, CA) into the pSP72-PGKneo plasmid. The "short arm" of homology from the mouse Beer gene locus was generated by amplifying a 2.4 kb fragment from the BAC clone 15G5. The 3' end of the fragment coincided with the translational initiation site of the Beer gene, and the anti-sense primer used in the PCR also included 30 ntd complementary to the 5' end of the β -galactosidase gene so that its 20 coding region could be fused to the Beer initiation site in-frame. The approach taken for introducing the "short arm" into the pSP72-βgal-PGKneo plasmid was to linearize the plasmid at a site upstream of the β -gal gene and then to co-transform this fragment with the "short arm" PCR product and to select for plasmids in which the PCR product was integrated by homologous recombination. The sense primer for the "short arm" amplification included 30 ntd complementary to the pSP72 vector to allow for this recombination event. The sequence of the sense primer was 5'-ATTTAGGTGACACT ATAGAACTCGAGCAGCTGAAGCTTAACCACATGGTGGCTCACAACCAT-3' (SEQ ID NO:36) and the sequence of the anti-sense primer was 5'-

30 AACGACGCCAGTGAATCCGTA
ATCATGGTCATGCTGCCAGGTGGAGGAGGGCA-3' (SEQ ID NO:37).

The "long arm" from the *Beer* gene locus was generated by amplifying a 6.1 kb fragment from BAC clone 15G5 with primers which also introduce the rarecutting restriction enzyme sites SgrAI, FseI, AscI and PacI. Specifically, the sequence of the sense primer was 5'-ATTACCACCGGTGACACCCGCTTCCTGACAG-3' (SEQ ID NO:38); the sequence of the anti-sense primer was 5'-

ATTACTTAATTAAACATGGCGCGCCAT

ATGGCCGGCCCCTAATTGCGGCGCATCGTTAATT-3' (SEQ ID NO:39) The resulting PCR product was cloned into the TA vector (Invitrogen, Carlsbad, CA) as an intermediate step.

The mouse Beer gene targeting construct also included a second selectable marker, the herpes simplex virus I thymidine kinase gene (HSVTK) under the control of rous sarcoma virus long terminal repeat element (RSV LTR). Expression of this gene renders mammalian cells sensitive (and inviable) to gancyclovir; it is therefore a convenient way to select against neomycin-resistant cells in which the construct has integrated by a non-homologous event (US Patent 5,464,764). RSVLTR-HSVTK cassette was amplified from pPS1337 using primers that allow subsequent cloning into the FseI and Ascl sites of the "long arm"-TA vector plasmid. For this PCR, the sequence of the sense primer ATTACGGCCGCCAAAGGAATTCAAGA TCTGA-3' (SEQ ID NO:40); the sequence of the anti-sense primer 5'-ATTACGGCGCGCCCCTC was ACAGGCCGCACCCAGCT-3' (SEQ ID NO:41).

The final step in the construction of the targeting vector involved cloning the 8.8 kb SgrAI-AscI fragment containing the "long arm" and RSVLTR-HSVTK gene into the SgrAI and AscI sites of the pSP72-"short arm"-\(\beta\)gal-PGKneo plasmid. This targeting vector was linearized by digestion with either AscI or PacI before electroporation into ES cells.

EXAMPLE 10

25

30

35

20

15

ANTISENSE-MEDIATED BEER INACTIVATION

17-nucleotide antisense oligonucleotides are prepared in an overlapping format, in such a way that the 5' end of the first oligonucleotide overlaps the translation initiating AUG of the Beer transcript, and the 5' ends of successive oligonucleotides occur in 5 nucleotide increments moving in the 5' direction (up to 50 nucleotides away), relative to the Beer AUG. Corresponding control oligonucleotides are designed and prepared using equivalent base composition but redistributed in sequence to inhibit any significant hybridization to the coding mRNA. Reagent delivery to the test cellular system is conducted through cationic lipid delivery (P.L. Felgner, *Proc. Natl. Acad. Sci. USA 84*:7413, 1987). 2 ug of antisense oligonucleotide is added to 100 ul of reduced serum media (Opti-MEM I reduced serum media; Life Technologies, Gaithersburg MD) and this is mixed with Lipofectin reagent (6 ul) (Life Technologies,

Gaithersburg MD) in the 100 ul of reduced serum media. These are mixed, allowed to complex for 30 minutes at room temperature and the mixture is added to previously seeded MC3T3E21 or KS483 cells. These cells are cultured and the mRNA recovered. Beer mRNA is monitored using RT-PCR in conjunction with Beer specific primers. In addition, separate experimental wells are collected and protein levels characterized through western blot methods described in Example 4. The cells are harvested, resuspended in lysis buffer (50 mM Tris pH 7.5, 20 mM NaCl, 1mM EDTA, 1% SDS) and the soluble protein collected. This material is applied to 10-20 % gradient denaturing SDS PAGE. The separated proteins are transferred to nitrocellulose and the western blot conducted as above using the antibody reagents described. In parallel, the control oligonucleotides are added to identical cultures and experimental operations are repeated. Decrease in Beer mRNA or protein levels are considered significant if the treatment with the antisense oligonucleotide results in a 50% change in either instance compared to the control scrambled oligonucleotide. This methodology enables selective gene inactivation and subsequent phenotype characterization of the mineralized nodules in the tissue culture model.

SEQUENCES

Sequence ID No. 1: Human BEER cDNA (complete coding region plus 5'and 3'UTRs)

5

ORF

AGAGCCTGTGCTACTGGAAGGTGGCGTGCCCTCTGGGTGGTACCATGCAGCTCCGACTGGCCCTGTGTCTCGTCTGC CTGCTGGTACACACCCTTCCGTGTAGTGGAGGGCCAGGGGTGGCAGGCGTTCAAGAATGATGCCACGGAAAATCATCCC CGAGCTCGGAGAGTACCCCGAGCCTCCACCGGAGCTGGAGAACAACAAGACCATGAACCGGGGGGAGAACCGAGGGGGGG CTCCCCACCACCCTTTGAGACCAAAGACGTGTCCGAGTACAGCTGCCGCGAGCTGCACTTCACCGGCTACGTGACCGAT GCCCGGCCCTGAACCCGCGCCCCACATTTCTGTCCTCTGCGCGTGGTTTGATTGTTTATATTTCATTGTAAATGCCTGC AACCCAGGGCAGGGGCTGAGACCTTCCAGGCCCTGAGGAATCCCGGGCGCGAAAGGGCCCCCCCAGCCCGCAGGTG AGGGGTCCCACGGGGCAGGGGAGGGAATTGAGAGTCACAGACACTGAGCCACGCAGCCCCCCCTCTGGGGCCGCCTACCT TTGCTGGTCCCACTTCAGAGGAGGCAGAAATGGAAGCATTTTCACCGCCCTGGGGTTTTAAGGGAGCGGTGTGGGAGTGG GAPAGTCCAGGGACTGGTTPAGPPAGTTGGATAAGPTTCCCCCTTGCACCTCGCTGCCCATCAGPPAGCCTGAGGCGTGC CCAGAGCACAAGACTGGGGGCAACTGTAGATGTGGTTTCTAGTCCTGGCTCTGCCACTAACTTGCTGTGTAACCTTGAAC TACACAATTCTCCTTCGGGACCTCAATTTCCACTTTGTAAAATGAGGGTGGAGGTGGGAATAGGATCTCGAGGAGCTAT CAGTTGCATTGATTCAGTGCCAAGGTCACTTCCAGAATTCAGAGTTGTGATGCTCTCTTCTGACAGCCAAAGATGAAAAA CAAACAGAAAAAAAAAAAGTAAAGAGTCTATTTATGGCTGACATATTTACGGCTGACAAACTCCTGGAAGAAGCTATGCTG CTTCCCAGCCTGGCTTCCCCGGATGTTTGGCTACCTCCACCCCTCCATCTCAAAGAAATAACATCATCCATTGGGGTAGA $\tt AGCCATCACACAGACCAGCACATCCCTTTTGAGACACCGCCTTCTGCCCACCACTCACGGACACATTTCTGCCT$ AGAAAACAGCTTCTTACTGCTCTTACATGTGATGGCATATCTTACACTAAAAGAATATTATTGGGGGAAAAACAAGT GCTGTACATATGCTGAGAAACTGCAGAGCATAATAGCTGCCACCCAAAAATCTTTTTGAAAATCATTTCCAGACAACCTC ${\tt GGTCGTTTTTTTGGCAATTCTTCCACGTGGGACTTGTCCACAAGAATGAAAGTAGTGGTTTTTAAAGAGTTAAGTTACAT}$ ATTTATTTTCTCACTTAAGTTATTTATGCAAAAGTTTTTCTTGTAGAGAATGACAATGTTAATATTGCTTTATGAATTAA CAGTCTGTTCTTCCAGAGTCCAGAGACATTGTTAATAAAGACAATGAATCATGACCGAAAG

35

30

Sequence ID No. 2: Human BEER protein (complete sequence)

MQLFLALGLVCLLVHTAFRVVEGQSWQAFKNDATEIIFELGEYPEFFFELENNKTMNRAENGGREFHHFFETHDVSEYSQ RELHFTRYVTDGEGRSAKEVTELV<u>GSGQC</u>GFARLLENAIGRGKMWRFSGFDFRQIEDRYRAQRVQLLGFGGEAFRAREVR LVASCKCKRLTRFHNQSELKDFGTEAARPQKGRKPRPRARSAKANQAELENAY

Sequence ID No. 3: Human Beer cDNA containing Sclerosteosis nonsense mutation

CTGCTGGTACACACAGCCTTCCGTGTAGTGGAGGGCTAGGGGTGGCAGGGGTTCAAGAATGATGCCACGGAAATCATCC: CGAGCTCGGAGAGTACCCGGAGCCTCCACCGGAGCTGGAGAACAACAACATGAACCGGGGGGGAGAACGAGGGGGGG CTCCCCACCACCCCTTTGAGACCAARGACGTGTCCGAGTACAGCTGCCGCGAGCTGCACTTCACCCGCTACGTGACCGAT 15 CATCGGCCGCGCAAGTGGTGGCGACCTAGTGGGCCCGACTTCCGCTGCATCCCGACCGCTACCGCGCAGCGCAGCGCGTGC AGCTGCTGTGCCGGTGGTGAGGCGCGCGCGCGCGCGCAAGGTGCGCCTGGTGGCCTCGTGCAAGTGCAAGCGCCTCACC CGCTTCCACAACCAGTCGGAGCTCAAGGACTTCGGGACCGAGGCCGCTCGGCCGCAGAAGGGCCGGAAGCCGCGGGCCCCG 20 ${\tt GCCCCGGCCCTGAACCCGCGCCCCACATTTCTGTCCTCTGCGCGTGGTTTGATTGTTTATATTTCATTGTAAATGCCTGC}$ AACCCAGGGCAGGGGCTGAGACCTTCCAGGCCCTGAGGAATCCCGGGCCGCCAAGGCCCCCCTCAGCCCGCCAGCTG GAAAGTCCAGGGACTGGTTAAGAAAGTTGGATAAGATTCCCCCTTGCACCTCGCTGCCCATCAGAAAGCCTGAGGCGTGC 25 TACACAATTCTCCTTCGGGACCTCAATTTCCACTTTGTAAAATGAGGGTGGAAGGGAATAGGATCTCGAGGAGACTAT CAAACAGAAAAAAAAAAAGAGTATATTATGGCTGACATATTTACGGCTGACAAACTCCTGGAAGAAGCTATGCTG 30 $\tt CTTCCCAGCCTGGCTTCCCCGGATGTTTGGCTACCTCCACCCCTCCATCTCAAAGAAATAACATCATCCATTGGGGTAGA$ AGCCATCACAAACTCACAGACCAGCACATCCCTTTTGAGACACCGCCTTCTGCCCACCACCACTCACGGACACATTTCTGCCTAGAAAACAGCTTCTTACTGCTCTTACATGTGATGGCATATCTTACACTAAAAGAATATTATTGGGGGAAAAACTACAAGT35 GCTGTACATATGCTGAGAAACTGCAGAGCATAATAGCTGCCACCCAAAAATCTTTTTGAAAATCATTTCCAGACAACCTC

TTAUTTTOTGTGTAUTTTTAATTGTTAALABALALARGTTTTAAACAGRAGCACATGACATATGAARGCCTGCAGGAUT GGTCGTTTTTTTGGCAATTCTTCCACGTGGGACTTGTCCACAAGAATGAAAAGTAGTGGTTTTTAAAGAGTTAAGTTAUTT ATTTATTTTCTCACTTAAGTTATTTATGCAAAAGTTTTTCTTGTAGAGAATGACAATGTTAATATTGCTTTATGAATTAA CAGTCTGTTCTTCCAGAGTCCAGAGACATTGTTAATAAAGACAATGATGACCGAAAG

5

Sequence ID No. 4: Truncated Human Beer protein from Sclerosteosis

MQLFLALCEVOLLVHTAFRVVEG*

10

Sequence ID No. 5: Human BEER cDNA encoding protein variant (V10I)

AGRECCTGTGCTACTGGARGGTGGCGTGCCCTCTGTGGCTGGTACCATGCAGCTCCCACTGGCCCTGTGTCTCATCTGC 15 $\tt CTGCTGGTACACAGCCTTCCGTGTAGTGGAGGGCCAGGGGTGGCAGGGGTTCAAGAATGATGCCACGGAAATCATCCG$ CGAGCTCGGAGAGTACCCCGAGCCTCCACCGGAGCTGGAGAACAACAAGACCATGAACCGGGCGGAGAACGGGAGGAGGGCGG CTCCCCACCACCCCTTTGAGACCAAAGACGTGTCCGAGTACAGCTGCCGGAGCTGCACTTCACCCGCTACGTGACCGAT GGGCCGTGCCGCAAGCCGGTCACCGAGCTGGTGTGCTCCGGCCAGTGCGGCCCGGCGCGCTGCTGCCCAACGC CATCGGCCGCGGCAAGTGGTGGCGACCTAGTGGGCCCGACTTCCGCTGCATCCCGGACCGCTACCGCGCGCAGCGCTGC 20 AGCTGCTGTGTCCCGGTGGTGAGGCGCCGCGCGCGCAAGGTGCGCCTGGTGGCCTCGTGCAAGTGCAAGCGCCTCACC CGCTTCCACAACCAGTCGGAGCTCAAGGACTTCGGGGACCGAGGCCGCTCGGCCGCAGAAGGGCCGGAAAGCCGCGGCCCCG GCCCCGGCCCTGAACCCGCGCCCACATTTCTGTCCTCTGCGCGTGGTTTGATTGTTTATATTTCATTGTAAATGCCTGC AACCCAGGGCAGGGGCTGAGACCTTCCAGGCCCTGAGGAATCCCGGGCGGCCAAGGCCCCCCTCAGCCCGCCAGCTG 25 AGGGGTCCCACGGGGCAGGGGAATTGAGAGTCACAGACACTGAGCCACGCAGCCCGCCTCTGGGGCCGCCTACCT TTGCTGGTCCCACTTCAGAGGGAGGCAGALATGGAAGCATTTTCACCGCCCTGGGGTTTTAAGGGAGCGGTGTGGGAGTGG ${\tt GAAAGTCCAGGGACTGGTTAAGAAAGTTGGATAAGATTCCCCCTTGCACCTCGCTGCCCATCAGAAAGCCTGAGGCGTGC}$ $\tt CCAGAGCACAAGACTGGGGGCAACTGTAGATGTGGTTTCTAGTCCTGGCTCTGCCACTAACTTGCTGTAACCTTGAAC$ 30 TACACAATTCTCCTTCGGGACCTCAATTTCCACTTTGTAAAATGAGGGTGGAGGTGGGAATAGGATCTCGAGGAGACTAT $\tt CTTCCCAGCCTGGCTTCCCCGGATGTTTGGCTACCTCCACCCCTCCATCTCAAAGAAATAACATCATCCATTGGGGTAGA$

f l0 Sequence ID No. 6: Human BEER protein variant (V10I)

MQLFLALCLICLLVHTAFRVVEGQGWQAFKNDATEIIRELGEYFEFFFELENNKTMNRAENGGRFEHHFFETKDVSEYSC RELHFTRYVTDGFCRSAKFVTELVCSGQCGFARLLFNAIGRGKWWRFSGFDFRCIFDRYRAQRVQLLCFGGEAFRARKVR LVASCKCKRLTRFHNOSELKDFGTEAARFQKGRKFRFRARSAKANOAELENAY

15

Sequence ID No. 7: Human Beer cDNA encoding protein variant (P38R)

20 CGAGCTCGGAGAGTACCCCGAGCCTCCACCGGAGCTGGAGAACAACAAGACCATGAACCGGGGGAGAACGGAGGAGGGCGGC CTCCCCACCACCCCTTTGAGACCAAAGACGTGTCCGAGTACAGCTGCCGCGAGCTGCACTTCACCCGCTACGTGACCGAT CATCGGCCGCGCAAGTGGTGGCGACCTAGTGGGCCCGACTTCCGCTGCATCCCCGACCGCTACCGCGCGCACCGCGTGC AGCTGCTGTGTCCCGGTGGTGAGGCGCCGCGCGCGCGCAAGGTGCGCCTCGTGCCAAGTGCAAGCGCCTCACC CGCTTCCACAACCAGTCGGAGCTCAAGGACTTCGGGACCGAGGGCCGCTCGGCCAGAAAGGGCCGGAAGCCGCGCCCCG GCCCCGGCCCTGAACCCGCGCCCCACATTTCTGTCCTCTGCGCGTGGTTTGATTGTTATATTTCATTGTAAATGCCTGC 30 AACCCAGGGCAGGGGCTGAGACCTTCCAGGCCCTGAGGAATCCCGGGCGCCAGGCCAAGGCCCCCTCAGCCCGCCAGCTG $\tt AGGGGTCCCACGGGGCAGGGGAGTGAGAGTCACAGACACTGAGCCACGCAGCCCGGCTCTGGGGCCGCCTACCT$ TTGCTGGTCCCACTTCAGAGGAGGCAGAAATGGAAGCATTTTCACCGCCCTGGGGTTTTAAGGGAGCGGTGTGGGAGTGG GAAAGTCCAGGGACTGGTTAAGAAAGTTGGATAAGATTCCCCCTTGCACCTCGCTGCCCATCAGAAAGCCTGAGGCGTGC CCAGAGCACAAGACTGGGGGCAACTGTAGATGTGGTTTCTAGTCCTGGCTCTGCCACTAACTTGCTGTAACCTTGAAC TACACAATTCTCCTTCGGGACCTCAATTTCCACTTTGTAAAATGAGGGTGGAGGTGGGAATAGGATCTCGAGGAGACTAT 35

15 Sequence ID No. 8: Human Beer protein variant (P38R)

MQLPLALCLVCLLVHTAFRVVEGQGWQAFKNDATEIIRELGEYFEFFFELENNKTMNRAENGGRFFHHFFETKDVSEYSC RELHFTRYVTDGFCRSAKFVTELVCSGQCGFARLLFNAIGRGKWWRFSGFDFRCIFDRYRAQRVQLLCFGGEAFRARKVR LVASCKCKRLTRFHNQSELKDFGTEAARFQKGRKFRFRARSAKANQAELENAY

20

Sequence ID No. 9: Vervet BEER cDNA (complete coding region)

35

Sequence ID No. 10: Vervet BEER protein (complete sequence)

MQLFLALCLVCLLVHAAFRVVEGQGWQAFKNDATEIIFELGEYFEFFFELENNKTUNRAENGGRFFHHFFETYDVSEYSC RELHFTRYVTDGFCRSAKFVTELVGSGQCGFARLLFNAIGRGKWWRFSGFDFRCIFDRYRAQRVQLLCFGGAAFRARKVR LVASCKCKRLTRFHUQSELKDFGFEAARFQKGRKFRFRARGAHANQAELENAY

5

Sequence ID No. 11: Mouse BEER cDNA (coding region only)

20 Sequence ID No. 12: Mouse BEER protein (complete sequence)

MQFSLAFCLICLLVHAAFCAVEGQGWQAFRNDATEVIFGLGEYFEFFFENNQTMNRAENGGREFHHFYDAKDVSEYSCRE LHYTRFLTDGFCRSAKEVTELVCSGQCGEARLLFNAIGRVKWWRENGFDFRCIFDRYRAQRVQLLCFGGAAFRSRKVRLV ASCKCKRLTRFHNQSELKDFGFETARFQKGRKPRFGARGAKANQAELENAY

25

Sequence ID No. 13: Rat BEER cDNA (complete coding region plus 5' UTR)

5 Sequence ID No. 14: Rat BEER protein (complete sequence)

MQLSLAFCLACLLVHAAFVAVESQGWQAFKHDATEIIFGLREYFEFFQELENNQTMURAENGGREFHHFYDTHDVSEYSS RELHYTRFYTDGECRSAKEVTELVCSGQCGEARLLENAIGRVKWWRENGEDERCIEDRYRAQRVQLLCEGGAAFESREUK LVASCECKRLTRFHNQSELKDFGFETARFQKGRKERFRARGARANQAELENAY

10

Sequence ID No. 15: Bovine BEER cDNA (partial coding sequence)

- Sequence ID No. 16: Bovine BEER protein (partial sequence -- missing signal sequence and last 6 residues)

NDATEIIFELGEYFEFLFELNNKTKNRAENGGREFHHFFETKDASEYSCRELHFTRYVTDGFCRSAKFVTELVCSGQCGF ARLLENAIGRGKWWRFSGFDFRCIFDRYRAQRVQLLCFGGAAFRARKVRLVASCKCKRLTRFHNQSELKDFGFEAARFQT GRKLRFRARGTKASRA

30

Sequence ID No. 17: MluI - AviII restriction fragment used to make mouse Beer transgene

35

 $\tt CGCGTTTTGGTGAGCAGCAATATTGCGCTTCGATGAGCCTTGGCGTTGAGATTGATACCTCTGCTGCACAAAAGGCAATC$

GACCGAGCTGGACCAGCGCATTCGTGACACGGTCTCCTTCGAACATTATTCGCAATGGAGTGTCATCATCAAGGACNGCC TGATCCCAAATGGTGCTATCCACGCAGCGGCAATCGAAAACCCTCAGCCGGTGACCAATATCTACAACATCAGCCTTGGT ATCCTGCGTGATGAGCCAGCGCAGAACAAGGTAACCGTCAGTGCCGATAAGTTCAAAGGTAAACCTGGTGTTGATACCAA CATTGAMACGTTGATCGAMAMACGCGCTGAMAMACGCTGCTGMATGTGCGGGGGGGTGGATGTCACAMAGCAMATGGCAGCAG ACAAGAAAGCGATGGATGAACTGGCTTCCTATGTCCGCACGGCCATCATGATGGAATGTTTCCCCGGTGGTGTTATCTGG CAGCAGTGCCGTCGATAGTATGCAATTGATAATTATTATCATTTGCGGGTTCCTTTCCGGCGATCCGCCTTGTTACGGGGC GGCGACCTCGCGGGTTTTCGCTATTTATGAAAATTTTCCGGTTTAAGGCGTTTCCGTTCTTCTTCGTCATAACTTAATGT TTTTATTTAAAATACOCTCTGAAAAGAAAGGAAACGACAGGTGCTGAAAGGGAGCTTTTTGGCCTCTGTCGTTTCCTTTC TCTGTTTTTGTCCGTGGAATGAACAATGGAAGTCAACAAAAAGCAGAGCTTATCGATGATAAGCGGTCAAACAATGAGAAAT TOGCGGCCGCATARTACGACTCACTATAGGGATCGACGCCTACTCCCCGGCATGRAGCGGAGGAGCTGGACTCCGCATG 10 CCCAGAGACGCCCCCAACCCCCAAAGTGCCTGACCTCAGCCTCTACCAGCTCTGGCTTGGGCTTGGGCGGGGTCAAGGC TACCACGTTCTCTTAACAGGTGGCTGGGCTGTCTTTGGCCGCGCGTCATGTGACAGCTGCCTAGTTCTGCAGTGAGGTC ACCGTGGAATGTCTGCCTTCGTTGCCATGGCAACGGGATGACGTTACAATCTGGGTGTGGAGCTTTTCCTGTCCGTGTCA GGRALTOGALATACCCTARLATACCCTAGALGAGGALGTAGCTGAGCCLAGGCTTTCCTGGCTTCTCCAGATALAGTTTG ACTTRGATGGAAAAAAAAACAAAATGATAAAGACCCGAGCCATCTGAAAATTTCCTCCTAATTGCACCACTAGGAAATGTGTA 15 TATTATTGAGCTCGTATGTGTTCTTATTTTAAAAAGAAAACTTTAGTCATGTTATTAATAAGAATTTCTCAGCAGTGGGA GAGAACCAATATTAACACCAAGATAAAAGTTGGCATGATCCACATTGCAGGAAGATCCACGTTGGGTTTTCATGAATGTG AAGACCCCATTTATTAAAGTCCTAAGCTCTGTTTTTGCACACTAGGAAGCGATGGCCGGGATGGCTGAGGGGCTGTAAGG ATCTTTCAATGTCTTACATGTGTGTTTCCTGTCCTGCACCTAGGACCTGCTGCCTAGCCTGCAGAGCCAGAGGGGGTT 20 ${\tt TCACATGATTAGTCTCAGACACTTGGGGGCAGGTTGCATGTACTGCATCGCTTATTTCCATACGGAGCACCTACTATGTG}$ TCAAACACCATATGGTGTTCACTCTTCAGAACGGTGGTGGTCATCATGGTGCATTTGCTGACGGTTGGATTGGTGGTAGA GAGCTGAGATATATGGACGCACTCTTCAGCATTCTGTCAACGTGGCTGTGCATTCTTGCTCCTGAGCAAGTGGCTAAACA A GAAGCCAGGGGGCTTGGCGGTCTCAGGAGCCTGCTTGCTGGGGGACAGGTTGTTGAGTTTTATCTGCAGTAGGTTGCCT25 GCTGCTCAGCTGGGAGGATCAACTGCATACCTAAAGCCAAGCCTAAAGCTTCTTCGTCCACCTGAAACTCCTGGACCAAG ${\tt GGGAGGTGGGGGGGAGAGCCTTTGCAGCTCTTTCCTCCCATCTGGACAGCGCTCTGGCTCAGCAGCCCATATGAGCACAGGC}$ ${\tt TATCCTCTCTAGGTAGACAGGACTCTGCAGGAGACACTGCTTTGTAAGATACTGCAGTTTAAATTTGGATGTTGTGAGG}$ 30 ${\tt GGAAAGCGAAGGGCCTCTTTGACCATTCAGTCAAGGTACCTTCTAACTCCCATCGTATTGGGGGGGCTACTCTAGTGCTAGTGCTAGTCTAGTGCTAGTAGTGCTAGTGCTAGTGCTAGTGCTAGTGCTAGTGCTAGTGCTAGTGCTAGTGCTAGTGCTAGTG$ CAPAGAACTGACAGACCGAAGCCTTGGAATATAAACACCAPAGCATCAGGCTCTGCCAACAGAACACTCTTTAACACTCA GGCCCTTTAACACTCAGGACCCCCACCCCCACCCCAAGCAGTTGGCACTGCTATCCACATTTTACAGAGAGGAAAAACTA 35 ${\tt GGCACAGGACGATATAAGTGGCTTGCTTAAGCTTGTCTGCATGGTAAATGGCAGGGCTGGATTGAGACCCAGACATTCCA}$

ACTOTAGGGTCTATTTTTTTTTTTTTCTCGTTGTTCGAATCTGGGTCTTACTGGGTAAACTCAGGCTAGCCTCACACTCAT TOOTCACAACATAGGAATTGTGATAGCAGCACACACACCGGAAGGAGCTGGGGAAATCCCACAGAGGGCTCCGCAGATG ACAGGCGAATGCCTACACAGAAGGTGGGGÁAGGGAAGCAGGGAACAGCATGGGCGTGGGACCACAAGTCTATTTGGGG 5 TACGGGCTCCTTATTGCCAAGAGGCTCGGATCTTCCTCCTCCTCCTTCCGGGGGTGCCTGTTCATTTTCCACCACTG CCTCCCATCCAGGTCTGTGGCTCAGGACATCACCCAGCTGCAGAAACTGGGCATCACCCAGGTCCTGAATGCTGCCGAGG SCAGGTCCTTCATGCACGTCAACACCAGTGCTAGCTTCTACGAGGATTGTGGCATCACCTACTTGGGCATCAAGGCCAAC GATACGCAGGAGTTCAACCTCAGTGCTTACTTTGAAAGGGCCACAGATTTCATTGACCAGGCGCTGGCCCATAAAATGG TRAGGRACGTACATTCCGGCACCCATGGAGCGTARGCCCTCTGGGACCTGCTTCCTCCARAGAGGCCCCCACTTGARRAR 10 GGTTCCAGRARGATCCCARRATATGCCACCARCTAGGGATTARGTGTCCTACATGTGRGCCGATGGGGGCCACTGCATAT STOTTCRATCGTTCCCCACCCACCTTATTTTTTGAGGCAGGGTCTCTTCCCTGATCCTGGGGCTCATTGGTTTATCTAG SOTGCTGGCCAGTGAGCTCTGGAGTTCTGCTTTTCTCTACCTCCCTAGCCCTGGGACTGCAGGGGCATGTGCTGGGCCAG 15 SCTTTTATGTCGCGTTGGGGATCTGAACTTAGGTCCCTAGGCCTGAGCACCGTAAAGACTCTGCCACATCCCCAGCCTGT TTGAGCAAGTGAACCATTCCCCCAGAATTCCCCCAGTGGGGCTTTCCTACCCTTTTATTGGCTAGGCATTCATGAGTGGTC ACCTCGCCAGAGGAATGAGTGGCCACGACTGGCTCAGGGTCAGCAGCCTAGAGATACTGGGTTAAGTCTTCCTGCCGCTC GCTCCCTGCAGCCGCAGACAGAAAGTAGGACTGAATGAGAGCTGGCTAGTGGTCAGACAGGACAGAAGGCTGAGAGGGTC ACAGGGCAGATGTCAGCAGAGCAGACAGGTTCTCCCTCTGTGGGGGAGGGGTGGCCCACTGCAGGTGTAATTGGCCTTCT 20 TTGTGCTCCATAGAGGCTTCCTGGGTACACAGCAGCTTCCCTGGTGATTCCCAAAGAGAAACTCCCTACCACTGGA $\tt CTTACAGAAGTTCTATTGACTGGTGTAACGGTTCAACAGCTTTGGCTCTTGGTGGACGGTGCATACTGCTGTATCAGCTC$ GCTCAGTGACTGGGCATTTCTGAACATCCCTGAAGTTAGCACACATTTCCCTCTGGTGTTCCTGGCTTAACACCTTCTAA ATCTATATTTTATCTTTGCTGCCCTGTTACCTTCTGAGAAGCCCCTAGGGCCACTTCCCTTCGCACCTACATTGCTGGAT 25 GGTTTCTCTCCTGCAGCTCTTAAATCTGATCCCTCTGCCTCTGAGCCATGGGAACAGCCCAATAACTGAGTTAGACATAA ${\tt TCCTTCACATGGCACGAGTATGAAGCTTTATTACAATTGTTTATTGATCAAACTAAACTCATAAAAAGCCAGTTGTCTTTC}$ ACCTGCTCAAGGAAGGAACAAAATTCATCCTTAACTGATCTGTGCACCATTGCACAATCCATACGAATATCTTAAGAGTAC ${\tt TALGATTTTGGTTGLGAGAGTCACATGTTACAGALATGTACAGCTTTGLCLLAGGTGCATCCTTGGGLAGCGLAGTGACCT}$ GCTGTTCCAGCCCCCTACCTTCTGAGGCTGTTTTGGAAGCAATGCTCTGGAAGCAACTTTAGGAGGTAGGATGCTGGAAC ${\tt AGCGGGTCACTTCAGCATCCCGATGACGAATCCCGTCAAAGCTGTACATTCTGTAACAGACTGGGAAAGCTGCAGACTTT}$ ARGCCAGGGCCTATGGTCCCTCTTAATCCCTGTCACACCCARCCGAGCCCTTCTCCCAGCCGTTCTGTGCTTCTC CCTCATTCAGGGAACTCTGGGGCATTCTGCCTTTACTTCCTCTTTTTGGACTACAGGGAATATATGCTGACTTGTTTTGA CCTTGTGTATGGGGAGACTGGATCTTTGGTCTGGAATGTTTCCTGCTAGTTTTTCCCCATCCTTTGGCAAACCCTATCTA

30

35

TATOTTACCACTAGGCATAGTGGCCCTCSTTCTGGGGCCTTGGCTTCAGGCTGGTTCTCGGGGAAC (ATGTCCCTGGTTTCT CCCCAGCATATGGTGTTCACAGTGTTCACTGCGGGTGGTTGCTGAACARAGCGGGGGATTGCATCCCAGAGCTCCGGTGCC TTGTGGGTACACTGCTAAGATAARATGGATACTGGCCTCTCTGACCACTTGCAGAGGTCTGGTGCUTTGTGGGTACAC TGCTAAGATAAAATGGATACTGGCCTCTCTATCCACTTGCAGGACTCTAGGGAACAGGAATCCATTACTGAGAAAAACC AGGGGCTAGGAGCAGGGAGGTAGCTGGGCAGCTGAAGTGGCGACTAACCAATGAATACCAGAGTTTGGATCTCTAG AATACTCTTAAAATCTGGGTGGGCAGAGTGGCCTGCCTGTAATCCCAGAACTCGGGGAGGCGAGACAGGGAAATCATCAGA TAAARCATTGAAGAAGACAGTAGATGCCAATTTTAAGCCCCCACACGTGCACAAGTGTGCGCTTTGARCACACATAT ATTAGAGTTCACAGGAAAGTGTGAGTGAGCACACCCATGCACACAGACATGTGTGCCAGGGAGTAGGAAAAGGAGCCTGGG 10 TTTGTGTATAAGAGGGAGCCATCATGTGTTTCTAAGGAGGGGGTGTGAAGGAGGCGTTGTGTGGGCTGGGACTGGAGCAT GGTTGTAACTGASCATGCTCCCTGTGGGAAACAGGAGGGTGGCCACCCTGCAGAGGGTCCCACTGTCCAGCGGGATCAGT ARANGCCCCTGCTGAGARCTTTAGGTRATAGCCAGAGAGAGARAGARAGGALAGTGGGGGACTCCCATCTCTGATGTAG GAGGATCTGGGCAAGTAGAGGTGCGTTTGAGGTAGAAAGAGGGGTGCAGAGGAGATGCTCTTAATTCTGGGTCAGCAGTT 15 TGCTGGAAATGGCCGAGCATCAACCCTGGCTCTGGAAGAACTCCATCTTTCAGAAGGAGAGTGGATCTGTGTATGGCCAG CGGGGTCACAGGTGCTTGGGGCCCCTGGGGGACTCCTAGCACTGGGTGATGTTTATCGAGTGCTCTTGTGTGCCAGGCAC 20 AGCTAGCGGCAAGGGTAGAGGGCGAGCTCCCTGTGCAGGAGALATGCAAGCAAGAGATGGCAAGCCAGTGAGTTAAGCAT TCCTTTCTTCCCACCATTGCTTTCCTTGTCCTTGAGAAATTCTGAGTTTCCACTTCACTGGTGATGCAGACGGAAACAGA 25 GTGTGTGCCTGCATGAGTTCATGTGTGCCACGTGTGTGCGGGAACCCTTGGAGGCCACAAGGGGCATCTGATCCCCTGG AACTGGAGTTGGAGGAGGTTGTGAGTCCCCTGACATGTTTGCTGGGAACTGAACCCCGGTCCTATGCAAGAGCAGGAAGT 30 GCAGTTATCTGCTGAGCCATCTCCAGTCCTGAAATCCATTCTCTTAAAATACACGTGGCAGAGACATGATGGGATTTA CGTATGGATTTAATGTGGCGGTCATTAAGTTCCGGCACAGGCAAGCACCTGTAAAGCCATCACCACAACCGCAACAGTGA ATGTGACCATCACCCCCATGTTCTTCATGTCCCCTGTCCCCTCCATCCTCCATCTCAAGCACCTCTTGCTCTGCCTCTG TCGCTGGAGAACAGTGTGCATCTGCACACTCTTATGTCAGTGAAGTCACACAGCCTGCACCCCTTCCTGGTCTGAGTATT 35 GTGTATGCACATGTGCCACATGTGTACAGATACTATGGAGGCCAGAAGAGGCCATGGCCGTCCCTGGAGCTGGAGTTACA

GGCAGCGTGTGAGCTGCCTGGTGGTGGTGGGAACCAAACCTTGAAACCAAGCACTTTTAACTACTACTACTACAGCAC TOTCAGTACCOTTCTTCATTTCTCCGCCTGGGTTCCATTGTATGGACACATGTAGCTAGAATATCTTGCTTATCTAATTA TGTACATTGTTTTGTGCTAAGAGAGAGTAATGCTCTATAGCCTGAGCTGGCCTCAATCTTGCCATCCTCCTGCCTCAGCC TCCTCCTCCTGAGTGCTAGGATGACAGGCGAGTGGTAACTTACATGGTTTCATGTTTTGTTCAAGACTGAAGGATAACAT TCATACAGAGARGETCTGGUTCACAAAGTGTGCAGTTCACTGAATGGCAGAACCGGTGATCAAGAAACAAAACTCAGGG CTGGAGAGATGGCACTGACTGCTCTTCCAGAGGTCCGGAGTTCAATTCCCAGGAACCACATGGTGGCTCACAGCCATCT ACACACACACACAAATTACCACCCAGAAAGCCCACTCCATGTTCCCTCCGCGACGTCTCTGCCTACAGTACTCCCCAGGTT ACCACTGTTCAGGCTTCTAACAACCTGGTTTACTTGGGCCTCTTTTCTGCTCTGTGGAGCCACACATTTGTGTGCCTCAT 10 CCATGCATGGCACAGTGTGTGGGGATGTCAGAGTATTGTGAACAGGGGACAGTTCTTTTCTTCAATCATGTGGGTTCCAG AGGTGGGGGGTTGTTCCATAGCCCAAACTGGCTTTGCACTTGCAGTTCAAAGTGACTGCCTGTCTCCACCTCTTAGAGTA TGAAGGGATGACTGGACTGGACATGAGCGTGGAAGCCRGAGAACAGCTTCAGTCTAATGCTCTCCCAACTGAGCTATTTC 15 GGTTTGCCAGAGAACAACTTACAGAAAGTTCTCAGTGCCATGTGGATTCGGGGTTGGAGTTCAACTCATCAGCTTGACAT TGGCTCCTCTACCCACTGAGCCTTCTCACTACTCTCTACCTAGATCATTAATTCTTTTTTAAAAAGACTTATTAGGGGGC TGGAGAGATGGCTCAGCCGTTAAGAGCACCGAATGCCCTTCCAGAGGTCCTGAGTTCAATTCCCAGCATGCCATTGCTGG GCAGTAGGGGGGCAGGTGTTCAACGTGAGTAGCTGTTGCCAGTTTTCCGCGGTGGAGAACCTCTTGACACCCTGCTGTT $\tt CCTGGTCATTCTGGGTGGGTGCATGGTGATATGCTTGTTGTATGGAAGACTTTGACTGTTACAGTGAAGTTGGGCTTCCA$ 20 CAGTTACCACGTCTCCCCTGTTTCTTGCAGGCCGGGTGCTTGTCCATTGCCGCGAGGGCTACAGCCGCTCCCCAACGCTA GTTATCGCCTACCTCATGATGCGGCAGAAGATGGACGTCAAGTCTGCTCTGAGTACTGTGAGGCAGAATCGTGAGATCGG CCCCAACGATGGCTTCCTGGCCCAACTCTGCCAGCTCAATGACAGACTAGCCAAGGAGGGCAAGGTGAAACTCTAGGGTG CCCACAGCCTCTTTTGCAGAGGTCTGACTGGGAGGGCCCTGGCAGCCATGTTTAGGAAACACAGTATACCCACTCCCTGC ACCACCAGACACGTGCCCACATCTGTCCCACTCTGGTCCTCGGGGGCCACTCCACCCTTAGGGAGCACATGAAGAAGCTC 25 ${\tt CCTAAGAAGTTCTGCTCCTTAGCCATCCTTTCCTGTAATTTATGTCTCTCCTGAGGTGAGGTTCAGGTTTATGTCCCTG}$ TCTGTGGCATAGATACATCTCAGTGACCCAGGGTGGGAGGGCTATCAGGGTGCATGGCCCGGGACACGGGCACTCTTCAT GACCCCTCCCCACCTGGGTTCTTCCTGTGTGGTCCAGAACCACGAGCCTGGTAAAGGAACTATGCAAACACAGGCCCTG ACCTCCCCATGTCTGTTCCTGGTCCTCACAGCCGGACACGCCCTGCTGAGGCAGACGAATGACATTAAGTTCTGAAGCAG 30 ${\tt AGATACTACATAGGGGCCCTTGGGTAAGCAAATCCATTTTTCCCAGAGGCTATCTTGATTCTTTGGAATGTTTAAAGTGT}$ GCCTTGCCAGAGAGCTTACGATCTATATCTGCTGCTTCAGAGCCTTCCCTGAGGATGGCTCTGTTCCTTTGCTTGTTAGA ${\tt CAAACAAACAAAGGACCTCCATTTGGAGAATTGCAAGGATTTTATCCTGAATTATAGTGTTGGTGAGTTCAAGTCATCAC}$ GCCAAGTGCTTGCCATCCTGGTTGCTATTCTAAGAATAATTAGGAGGAGGAACCTAGCCAATTGCAGCTCATGTCCGTGG 35 GTGTGTGCACGGGTGCATATGTTGGAAGGGGTGCCTGTCCCCTTGGGGACAGAAGGAAAAATGAAAGGCCCCTCTGCTCAC

DETGGCCATTTACGGGAGGCTCTGCTGGTTCCACGGTGTCTGTGCACGATCTTGAAAUTGACTCGCTGGAACAAAACGAG ACTTGGCGGCACCATGAGAATGGAGAGAGAGAGAGCAAAGAAACAGCCTTTAAAAGAACTTTCTAAGGGTGGTTTT TGAACCTCGCTGGACCTTGTATGTGTGCACATTTGCCAGAGATTGAACATAATCCTCTTGGGACTTCACGTTCTCATTAT TTGTATGTCTCCGGGGTCAGGCAGAGCCGTCAGCCACCACCACCAGGGCACATAGGCGTCTCATAAAAGCCCATTT ATATTTCAARTTCAGCTTTAAGTGTAAGACTCAGCAGTGTTCATGGTTAAGGTAAGGAACATGCCTTTTCCAGAGCTGCT GCAAGAGGCAGGAGAAGCAGACCTGTCTTAGGATGTCACTCCCAGGGTAAGACCTCTGATCACAGCAGGAGCAGAGCTG TGCAGCCTGGATGGTCATTGTCCCCTATTCTGTGTGACCACAGCAACCCTGGTCACATAGGGCTGGTCATCCTTTTTTT TTTTTTTTTTTTTTTTTTTGGCCCAGAATGAAGTGACCATAGCCAAGTTGTGTACCTCAGTCTTTAGTTTCCAAGCGGCT 10 CTCTTGCTCARTACAATGTGCATTTCAAAATAACACTGTAGAGTTGACAGAACTGGTTCATGTGTTATGAGAGAGGAAAA GAGAGGAAAGAACAAAACAAAACAAAACACACAAACCAAAACCAAAACATCTGGGCTAGCCAGGCATGATTGCAATGTUTACAG GCCCAGTTCATGAGAGGCAGAGACAGGAAGACCGCCGARAGGTCAAGGATAGCATGGTCTACGTATCGAGACTCCAGCCA GGGCTACGGTCCCAAGATCCTAGGTTTTGGATTTTGGGCTTTGGTTTTTGAGACAGGGTTTCTCTGTGTAGCCCTGGCTG TECTGGAACTCGCTCTGTAGACCAGGCTGGCCTCAAACTTAGAGATCTGCCTGACTCTGCCTTTGAGGGCTGGGACGAAT 15 GTAGAAGCAGTCTCAGGCCTGCTTGAGGCTGTTCTTGGCTTGGACCTGAAATCTGCCCCCAACAGTGTCCAAGTGCCA CATGACTTTGAGCCATCTCCAGAGAAGGAAGTGAAAATTGTGGCTCCCCAGTCGATTGGGACACAGTCTCTCTTTGTCTA 20 TTCTTCAGGTAAAATACCGATGTTGTGGAAAAGCCAACCCCGTGGCTGCCCGTGAGTAGGGGGTGGGGTTGGGAATCCTG $\tt CCTGCCTTTTCCAGGGGTAGGTCTGTTTTTTTGCTGTTCTATTGTCTTGAGAGCACAGACTAACACTTACCAAATGAGGG$ 25 ACCCGCCACCCCAAGTGGGTGTGGATAATGCCATGGCCAGCAGGGGGGCACTGTTGAGGCGGGTGCCTTTCCACCTTAAG TTGCTTATAGTATTTAAGATGCTAAATGTTTTAATCAAGAGAAGCACTGATCTTATAATACGAGGATAAGAGATTTTCTC30 GGATGGTGGGGTGAGGCAGAGCACTGTCACCTGCCAGGCATGGGAGGTCCTGCCATCCGGGAGGAAAAGGAAAGTTTAGC TCTCAGGCTGGCCTTGAACTTCTGATCGCCTGCCCCTGCCCCTGCCCCTGTCCCTGCCTCCAAGTGCTAGGACT AAAAGCACATGCCACCACACCAGTACAGCATTTTTCTAACATTTAAAAATAATCACCTAGGGGCTGGAGAGAGGGTTCCA 35

CTTTARABACCTCCTARAACCTRGCCCTGGRGGTACGRCTCTGGAARGCTGGCATACTGTGTAAGTTCATCTCATGGTG AGGGGGAGCACGGGGAACTTGGGCAGTGAAAATTCTTTTGCAGGACACTAGAGGAGGATAAATACCAGTCATTGCACCCAC TACTGGACAACTCCAGGGAATTATGCTGGGTGAAAAGAGAAAGGCCCCAGGTATTGGCTGCATTGGCTGCATTTGCGTAAC GACATGTGRACARCTCCATCAARRRGCGACAGRARAGRACGGGCTGTGGTGACAGCTACCTCTAATCTCCACCTCCGGGAG GACATCASGGCAGATCCTTGGGGCCF-AAGGCGGACAGGCGAGTCTCGTGGTAAAGGTCGTGTAGAAGCGGATGCATGAGCA 10 SGCTGTGGTGCTGGACTGGCATCTTTGGTGASCTGTSSASGGGAAATGGGTAGGGAGATCATAAAATCCCTCCGAATTAT GTGGTGTGCACCTATAGCCACGGGCACTTGGAAAGCTGGAAGAAGGATGGCGAGTTTGAAGGTATCTGGGGCTGTACA GCAAGACCGTCGTCCCCAAACCAAACCAAACAGCAAACCCATTATGTCACACAAGAGTGTTTATAGTGAGCGGCCTCGCT GAGAGCATGGGGTGGGGGTGGGGGACAGAATATCTAAACTGCAGTCAATAGGGATCCACTGAGACCCTGGGGC 15 TTGACTGCAGCTTAACCTTGGGGAAATGATAAGGGTTTTGTGTTGAGTAAAAGCATCGATTAACCTGACTTAACCTCAAATGA AGRARARGE TOROUGHARACARCARGE TOROUGH TOROUGH TOROUGH TARGE TOROUGH TOR TCTTCCGAAGGTCCAGAGTTCAAATCCCAGCAACCACATGGTGGCTCACAACCATCTGTAACGAGATATGATGCCCTCTT 20 CATGGGCCGAGGAGGAGAGAGAGATAGGCTGGTAAGCTCAGTTTCTGTATACCCCTTTTTCTTGTTGACACTACTTC AATTACAGATAAAATAACAAATAAACAAAATCTAGAGCCTGGCCACTCTCTGCTCGCTTGATTTTTCCTGTTACGTCCAG CAGGTGGCGGAAGTGTTCCAAGGACAGATCGCATCATTAAGGTGGCCAGCATAATCTCCCATCAGCAGGTGGTGCTGTGA GAACCATTATGGTGCTCACAGAATCCCGGGCCCAGGAGCTGCCCTCTCCCAAGTCTGGAGCAATAGGAAAAGCTTTCTGGC CCAGACAGGGTTAACAGTCCACATTCCAGAGCAGGGGARAAGGGACACAGAGACAAAAAGGGCCAGCTTCTAAC 25 AACTTCACAGCTCTGGTAGGAGAGATAGATCACCCCCAACAATGGCCACAGCTGGTTTTGTCTGCCCCGAAGGAAACTGA GTGTGGGTGACAGAAGATGAAAAAGGAGGACCCAGGCAGATCGCCACAGATGGACCGGCCACTTACAAGTCGAGGCAGGTG GCAGAGCCTTGCAGAAGCTCTGCAGGTGGACGACACTGATTCATTACCCAGTTAGCATACCACAGCGGGCTAGGCGGACC $\tt ACAGCCTCCTTCCCAGGGCTGGGGAGTCCTCCAACCTTCTGTCTCAGTGCAGCTTCCGCCAGCCCCTCC$ 30 TECTTTTGCACCTCAGGTGTGAACCCTCCCTCCCTCTCCCTGTGGCATGGCCCTCCTGCTACTGCAGGCTGAGCA $\tt ATGAGTTCGAATCCCCAGCAACCATGTGGAAAAATAACCTTCTAACCTCAGAGTTGAGGGGAAAGGCAGGTGGATTCTGG$ ${\tt GGGCTTACTGGCCAGCCTAACCTALLTGTCTCAGTCAGAGATCCTGTCTCAGGGAATAACTTGGGAGAATGA}$ 35

15

20

25

30

ATGAGGGARATGATTTTTTTGCTAAGARATGARATTTTGTGGTGGCGRAGARGCCTGGCCAGRAAGGAACTGCCTTG GCACACCAGCCTATAAGTCACCATGAGTTCCCTGGCTAAGAATCACATGTAATGGAGCCCAGGTCCCTCTTGCCTGGTGG TGGGGTCAATGGGATTCCTTTAAAGGCATCCTTCCCAGGGCTGGGTCATACTTCAATAGTAGGGTGCTTGCACAGCAAGC 5 GAGCAAACACCTTTAACTAAGACCATTAGCTGGCAGGGGTAACAAATGACCTTGGCTAGAGGAATTTGGTCAAGCTGGAT GGAGCCAGACAATTAAAAGCCAAGCTCATTTTGATATCTGAAAACCACAGCCTGACTGCCCTGCCGTGGGAGGTACTGG GAGAGOTG SCTGTGTCCCTGCCTCACCAACGCCCCCCCCCCAACACACTCCTCGGGTCACCTGGGAGGTGCCAGCAG 10 GGGCTTTARARAGGCARCCGTATCTAGGCTGGACACTGGAGCCTGTHCTACCGAGTGCCCTCCTCCACCTGGCAGCATGC AGCCCTCACTAGCCCCGTGCCTCATCTGCCTACTTGTGCACGCTGCCTTCTGTGCTGTGCAGGGCCAGGGGTGGCAAGCC TTCAGGAATGATGCCACAGAGGTCATCCCAGGGCTTGGAGAGTACCCCGAGGCCTCCTGAGAACCAGACCAGACCATGAA GGGGGGTCCTGGGAGGTGACTGGGGTGGTTTTAGCATCTTCTCAGAGGTTTGTGTGGGGTGGCTAGCCTCTGCTACATCA GGCCAGGGACACATTTGCCTGGAAGAATACTAGCACAGCATTAGAACCTGGAGGGCAGCATTGGGGGGGCTGGTAGAGAGC ACCCAAGGCAGGGTGGAGGCTGAGGTCAGCCGAAGCTGGCATTAACACGGGCATGGGCTTGTATGATGGTCCAGAGAATC TCCTCCTAAGGATGAGGACACAGGTCAGATCTAGCTGCTGACCAGTGGGGAAGTGATATGGTGAGGCTGGATGCCAGATG CCATCCATGGCTGTACTATATCCCACATGACCACCACATGAGGTALLGARGGCCCCAGCTTGARGATGGAGLLACCGAGA GGCTCCTGAGATALAGTCACCTGGGAGTAAGAAGAGCTGAGACTGGAAGCTGGTTTGATCCAGATGCAAGGCAACCCTAG ATTGGGTTTGGGTGGGAACCTGAAGCCAGGAGGAATCCCTTTAGTTCCCCCTTGCCCAGGGTCTGCTCAATGAGCCCAGA GGGTTAGCATTAAAAGAACAGGGTTTGTAGGTGGCATGTGACATGAGGGGGCAGCTGAGTGAAAATGTCCCCTGTATGAGCA CAGGTGGCACCACTTGCCCTGAGCTTGCACCCTGACCCCAGCTTTGCCTCATTCCTGAGGACAGCAGAAAACTGTGGAGGC CAGCTGGAGGGACACTCCAGAGAAATGACCTTGCTGGTCACCATTTGTGTGGGAGGAGAGCTCATTTTCCAGCTTGCCAC CACATGCTGTCCCTGTCTCCTAGCCAGTAAGGGATGTGGAGGAAAAGGCCCCCAAAGGAGCATGCAATGCAGTCA $\tt CGTTTTTGCAGAGGAAGTGCTTGACCTAAGGGCACTATTCTTGGAAAGGCCCCAAAACTAGTCCTTCCCTGGGCAAACAGG$ °CCTCCCCACATACCACCTCTGCAGGGGTGAGTAAATTAAGCCAGCACAGAAGGGTGGCAAGGCCTACACCTCCCCCT TTATGTCATATTGATCCTGACACCATGGAACTTTTGGAGGTAGACAGGACCCACACATGGATTAGTTAAAAGCCTCCCAT CCAACCCAATCTCCTTCCCCGGAGAACAGACTCTAAGTCAGATCCAGCCACCCTTGAGTAACCAGCTCAAGGTACACAGA

TTTGGGGCAAGTTGTTTTGTGAGGCTGGAGCTGTGATAATGAGGGGGGTTTGGAGGGGGGGTTTGGTGGGTTTUAAGTGT AATGAATTCTTATCCCTACCACCTGCCTTCTACCCCGCTCCTCCACAGAGCGGCTGTCCTGATTTATTACCTCCARTTAAC GTGTGGCTAGAGGCTACCAGGCAGGGCTGGGGGATGAGGCTAAACTGGAAGAGTGTTTGGTTAGTAGGCACAAAGCCTT 5 TTGAGGCCAGCCTGGGCTACATARAACCCAATCTCAARAGCTGUCAATTCTGATTCTGTGCCACGTAGTGCCUGATGTA ATRITIGATIGAASTOGTTGAATOOTGGGGCAACOTATTTTACAGATGTGGGGAAAAAGCAACTTTAAGTACCOTGCCCACA ATCTCACTGCCCCGGTGCCTCCTTCCTATAATCCATACAGATTCGAAAGCGCAGGGCAGGTTTGGAAAAAGAGAGAAA SPTGGAAGGAGCAGACCAGTCTGGCCTAGGCTGCAGCCCCTCACGCATCCCTCTCCCGCAGATGTGTCCGAAGTACAGCT 10 GOOGCGAGCTGCACTACACCCGCTTCCTGACAGACGGCCCATGCCGCAGCCCAAGCCGGTCACCGAGTTGGTGTGCTCC GBCCAGTGCGGCCCCCCCGCGCTGCCCCAACGCCATCGGGCGCGTGAAGTCGTGGCGCCCGAACGGACCGGATTTCCC GTCTGGTGGCCTCGTGCAAGTGCAAGCGCCTCACCCGCTTCCACAACCAGTCGGAAGTCAAGGACTTCGGGGCCGGAGACC 15 CTAGAGCGAGCCGGCGCTATGCAGCCCGGGGGATCCGATTCGTTTTCAGTGTAAAGCCTGCAGCCCAGGCCAGGGCT GCCALACTTTCCAGACCGTGTGGAGTTCCCAGCCCAGTAGAGACCGCAGGTCCTTCTGCCCGCTGCGGGGGAGGATGGGGGAGG GGSTGGGGTTCCCGCGGGCCAGGAGAGGAAGCTTGAGTCCCAGACTCTGCCTAGCCCGGGTGGGATGGGGATGTGTTTCTA CCCTCGCCGGACCTATACAGGACAAGGCAGTGTTTCCACCTTAAAGGGAAGGGAGTGTGGAACGAAAGACCTGGGACTGG TTATGGACGTACAGTAAGATCTACTCCTTCCACCCAAATGTAAAGCCTGCGTGGGCTAGATAGGGTTTCTGACCCTGACC 20 TEGCCACTGAGTGTGATGTTGGGCTACGTGGTTCTCTTTTGGTACGGTCTTCTTTGTAAAATAGGGACCGGAACTCTGCT TCAAATCTGCCTTCAAATCCATATCTGGGATAGGGAAGGCCAGGGTCCGAGAGATGGTGGAAGGGCCAGAAATCACACTC CTGGCCCCCGAAGAGCAGTGTCCCGCCCCAACTGCCTTGTCATATTGTAAAGGATTTTCTACACAACAGTTTAAGGT 25 ACACATTTCTGTCTAGAAACAGAGCGTCGTCGTGCTGTCCTCTGAGACAGCATATCTTACATTAAAAAGAATAATACGGG GGGGGGGGGGGGGGGGGCAAGTGTTATACATATGCTGAGAAGCTGTCAGGCGCCACAGCACCACCACAATCTTTTTGT 30 GTCATCTCACTCCCTTCCCTTGGTCACAAGACCCAAACCTTGACAACACCTCCGACTGCTCTGTAGCCCTTGTGGCA ATACGTGTTTCCTTTGAAAAGTCACATTCATCCTTTCCTTTGCAAACCTGGCTCATTCCCCAGCTGGGTCATCGTCAT 35 ACCCTCACCCAGCCTCCCTTTAGCTGACCACTCTCCACACTGTCTTCCAAAAGTGCACGTTTCACCGAGCCAGTTCCCT

GUTUGA GUTUGATUGGATTGCTCCTCCTTGCTCCAGACCCTTCTCCCACAAGATGTTCATCTCCCACTCCATCAAGCCCC AGGTTCATGGAACTCTTGCCTGCCCCTGAACCTTCCAGGACTGTCCCAGGGTCTGATGTGTCCTCTCTTGTAAAGCCC CACCCCACTATTTGATTCCCAATTCTAGATCTTCCCTTGTTCATTCCTTCACGGGATAGTGTCTCATCTGGCCAAGTCCT GCTTGATATTGGGATAAATGCAAAGCCAAGTACAATTGAGGACCAGTTCATCATTGGGCCCAAGCTTTTTCAAAAATGTGAA TTTTACACCTATAGAAGTGTAAAAAGCCTTCCAAAGCAAAGGCAATGCCTGGCTCTTCCATCAACATCAGGGCTCCTGCTT TATGGGTCTGGTGGGGTAGTACATTCATAAACCCAACACTAGGGGTGTGAAAGCAAGATGATTGGGAGTTCGAGGCCAAT CAACACTTTAAATCCAGTCAAGTGCATCTTTGCGTGAGGGGAACTCTATCCCTAATATAAGCTTCCATCTTGATTTGTGT ATGTGCACACTGGGGGTTGAACCTGGGCCTTTGTACCTGCCGGGCAAGCTCTCTACTGCTCTAAAACCCAGCCCTCACTGG 10 $\tt CTTTCTGTTTCAACTCCCAATGAATTCCCCCTAAATGAATTATCAATATCATGTCTTTGAAAAATACCATTGAGTGCTGCT$ GGTGTCCCTGTGGTTCCAGATTCCAGGAAGGACTTTTCAGGGAATCCAGGCATCCTGAAGAATGTCTTAGAGCAGGAGGG CAGGGTACTCAGGATTAARAAGCCTTCCCCCAARACAATTCCAAGATCAGTTCCTGGTACTTGCACCTGTTCAGCTATGCA GAGCCCAGTGGGCATAGGTGAAGACACCGGTTGTACTGTCATGTACTAACTGTGCTTCAGAGCCGGCAGAGACAAATAAT 15 GTTATGGTGACCCCAGGGGACAGTGATTCCAGAAGGAACACAGAAGAGAGTGCTGCTAGAGGCTGCCTGAAGGAGAAGAAGG GTCCCAGACTCTCTAAGCAAAGACTCCACTCACATAAAGACACAGGCTGAGCAGAGCTGGCCGTGGATGCAGGGAGGCCCA TCCACCATCCTTTAGCATGCCCTTGTATTCCCATCACATGCCAGGGGTGAGGGGCATCAGAGAGTCCAAGTGATGCCCAA 20 ${\tt AACAACAGGCTGATCTGGGAGGGGTACTCTATGGCAGGGAGCACGTGTGCTTGGGGTACAGCCAGACACGGGGCTTG}$ CACACACACACACACACACACACACACACACACCACTCACTTCTCACTCGAAGAGCCCCTACTTACATTCTAAGAACAAACC ATTCCTCCTCATAAAGGAGACAAAGTTGCAGAAACCCAAAAGAGCCACAGGGTCCCCACTCTCTTTGAAATGACTTGGAC TTGTTGCAGGGAAGACAGAGGGGTCTGCAGAGGCTTCCTGGGTGACCCAGAGCCACAGACACTGAAATCTGGTGCTGAGA CCTGTATAAACCCTCTTCCACAGGTTCCCTGAAAGGAGCCCACATTCCCCAACCTGTCTCCTGACCACTGAGGATGAGA ${\tt GCACTTGGGCCTTCCCCATTCTTGGAGTGCACCCTGGTTTCCCCATCTGAGGGCACATGAGGTCTCAGGTCTTGGGAAAG}$ TTCCACAAGTATTGAAAGTGTTCTTGTTTTGTTTGTTTAATTTAGGTGTATGAGTGCTTTTGCTTGAATATATGCCT 30 ${\tt TCACTGAGGTTCTTTCTGTGGCTAAAGAGACAGGAGACAAAGGAGAGTTTCTTTTAGTCAATAGGACCATGAATGTTCCT}$ ${\tt CGTAACGTGAGACTAGGGCAGGGTGATCCCCCAGTGACACCGATGGCCCTGTGTAGTATTAGCAGCTCTAGTCTTATTC}$ $\tt CTTAATAAGTCCCAGTTTGGGGCAGAGATATGTATTCCCTGCTTTGAAGTGGCTGAGGTCCAGTTATCTACTTCCAAGT$ 35 TTTCCCTGAGCAGTCAGGCCAGTCCAAAGCCCTTCAATTTAGCTTTCATAAGGAACACCCCTTTTGTTGGGTGGAGGTAG

CASTISUCTISAATOOCASCATTARGAAGGCAGAGACASTOSGATUTUTUFSAGTICACAGCCAGCCIGGTCTACGGAGC CARARROGRACARACAGARRARCAAGCCAGAGTGTTTGTCCCCGTATCTTATTAATCATATTTTTGTCCCTTTGCCATTT TAGACTARRAGACTCGGGRARGCAGGTCTCTCTCTTTCTCATCCGGRCACACCCAGARCCAGATGTATGGARGATGGC TAATGTGCTGCAGTTGCACATCTGGGGCTGGGTGGATTGGTTAGATGGCATGGGCTGGGTGTGGTTACGATGACTGCAG CATGUARGCAGAAGCCAAGGGACAGCCTTAGGGTAGTGTTTCCACAGACCCCCTCCCCCCTTTTAACATGGGCATCTCTCA TEGGUCTGGAGOTTGCCAACTGGGCTGGGCTGGCTAGCTTGTAGGTUUCAGGGGATOTGCATATCTCTGCCTCCCTAGTGC TGGGATTACAGTCATATATGAGCACACCTGGCTTTTTTATGTGGGTTCTGGGCTTTGAACCCAGATCTGAGTSCTTGCAA GPCAATCGGTTGAATGACTGCTTCATCTCCCCAGACCCTGGGATTCTACTTTCTATTAAAGTATTTCTATTAAAATA 10 AGCCCCTGCCCTGCACTCAGCAGTTCTTAGGCCTGCTGAGAGTCAAGTGGGGAGTGAGAGCAAGCCTCGAGACCCATC AGCGAAGCAGAGACAAAAAAAAAAACTTGGGATTCGAGGCTCGGGATTATGGAGATACAGAAAAGCGTCAGGAAAAGAAAA ATGARCCAGATGARTAGAGGCAGGARGGGTAGGGCCCTGCATACATGGARCCTGGTGTACATGTTATCTGCATGGGGTTT GCATTGCAATGGCTCTTCAGCAGGTTCACCACACTGGGAAACAGCAAAAAGAAGAAGAGTAGGTGGTGTTFGAGTCAGA TACTGTCAGTCATGCCTGAAGAAATGGAAGCAATTAACGATGCGCCGCAATTAGGATATTAGCTCCCTGAAGAAAGGCAA 15 GAAGCTGGGCTGTGGGCACTGAAGGGAGCTTTGAATGATGTCACATTCTCTGTATGCCTAGCAGGGCAGTATTGGAGACT GAGACTTGACTTGTGTGTCCATATGATTCCTCCTTTTCCTACAGTCATCTGGGGCTCCTGAGCTTCGTCCTTGTCCAAGA AGAGGACCACCGACCTCTGCCTGACAAAGCTGCAGGACCAGTCTCTCCTACAGATGGGAGACAGAGGCGAGAGATGA ATSGTCAGGGGAGGAGTCAGAGAAAGGAGGGAGAGGCAGAGACCAAAGGAGGGAAACACTTGTGCTCTACAGCTACTG 20 ACTGAGTACCAGCTGCGTGGCAGACAGCCAATGCCAAGGCTCGATCATGGCACCTCGTGGGACTCCTAGCCCAGTG TOTOTGTATCACCCTAGCTGTCCTGGAACTCACTCTGTAGACCAGGCTGGCCTCGAACTCAGAAATCCCCCCTGCCTCTGC 25 TATTATAATTCCAGGTTATAGTTCATTGCTGTAGAATTGGAGTCTTCATATTCCAGGTAATCTCCCACAGACATGCCACA AAACAACCTGTTCTACGAAATCTCTCATGGACTCCCTTCCCCAGTAATTCTAAACTGTGTCAAATCTACAAGAAATAGTG A CAGTCACAGTCTCTALACGTTTTGGGCATGAGTCTGAAGTCTCATTGCTALAGTACTGGGAAGATGAALACTTTACCTAGTGTCAGCATTTGGAGCAGAGCCTTTGGGATTTGAGATGGTCTTTTGCAGAGCTCCTAATGGCTACATGGAGAGAGGGGGCC TGGGAGAGACCCATACACCTTTTGCTGCCTTATGTCACCTGACCTGCTCCTTGGGAAGCTCTAGCAAGAAGGCCTTCCCT 30 GGATCACCCACCACCTTGCACCTCCAGAACTCAGAGCCAAATTAAACTTTCTTGTTACTGTCGTCAAAGCACAGTCGGTC GCGAGTAAGGTGTAAATGTTCATGGATGTAAATGGGCCCATATATGAGGGTCTGGGGTAACAAGAAGGCCTGTGAATATA 3.5

ATTGTGTGATT STGTGTGAGTCTGATGTCACATGCTCATCTTGCCCTATGAGTTGARRACCARATGGCCCCTGRGAGA TGCAGCAGACTACATATGCTCAGCCCTGAAGTCCTTCTAGGGTGCATGTCTCTCAGAATTTCAGAAAAGTCATCTGTGGC TCCAGGACUBCCTGCACTCTCCCCTCTGCCGCGAGGCTGCAGACTCTAGGCTGGGGTGGAAGCAACGCTTACCTCTGGGAC TCCTCCCGTTCACTTAGTTCTCAACAATAACTACTCTGAGAGCACTTATTAATAGGTGGCTTAGACATAAGCTTT33CTC TGGTGGCACTUTGGGAGTTCAAAGCCAGCCTGATCTACACAGCAAGCTCCAGGATATCCAGGGCAATGTTGGGAAAARCUT TTCTCARACELARGAGGGGTTCAGTTGTCAGGAGGAGGAGCCATGGGTTARGARGTCTAGACGAGCCATGGTGATGCATA 10 COTTTCATCOAAGCACTTAGGAGGCAAAGAAAGTGAAACTCTTTGACTTTGAGGCCAGCTAGGTTACATAGTGATACCC ACTCCCTAGAACTAGAGTCATAGACAGTTGTGACACTCCCCAACCCACCATGTGGGTGCTTGAAGCTAAACTCCTGT CCTTTGTAAAGCAGCAGGTGTCTATGAACCCTGAACCATCTCTCCAGTCTCCAGATGTGCATTCTCAAAGAGGAGTCCTT 15 CATATTTCCCTAPACTGAACATCCTTATCAGTGAGCATCCTCGAGTCACCAPAAGCTACTGCAPACCCTCTTAGGGAAACAT CARAGCATGCATGTACACCATTCTTATTAGACTATGCTTTGCTARAGACTTTCCTAGATACTTTARARACATCACTTCT GCCTTTTGGTGGGCAGGTTCCAAGATTGGTACTGGCGTACTGGAAACAAGGTAGAGATCTAGAAATCACAGCAGG TCRGARGGGCCAGCCTGTACARGAGAGAGTTCCACACCTTCCAGGARCACTGAGCAGGGGGGCTGGGACCTTGCCTCTCAG 20 CCCAAGAAACTAGTGCGTTTCCTGTATGCATGCCTCTCAGAGATTCCATAAGATCTGCCTTCTGCCATAAGATCTCCTGC AACTGATCTAGGGAGCTGGCTCAGCAGTTAAGAGTTCTGGCTGCCCTTGCTTCAGATCTTGCTTTGATTCCCAGCACCCA ${\tt CATGATGGCTTTCAACTGTATCTCTGCTTCCAGGGGATCCAACAGCCTCTTCTGACCTCCATAGACAAGACCTAGTCCTC}$ 25 ${\tt TGCAAGAGCACCAAATGCTCTTATCTGTTGATCCATCTCTAGCCTCATGCCAGATCATTTAAAACTACTGGACACTGT}$ TTTATAAGAAAGATATCTGCATTTGTCTCCTGAGAGAACAAAGGGTGGAGGGCTACTGAGATGGCTCTAGGGGTAAAGGT 30 $\tt CCTCAPACTTCCCACACATGTGCTGTGGCTTPTGTGTAACCCCAPTPPGTAPAGATAGTTTTPPACACTPCATPPGGTPG$ GGTTTCTTCATGACCCCAAGGAATGATGCCCCTGATAGAGCTTATGCTGAAACCCCATCTCCATTGTGCCATCTGGAAAG AGACAATTGCATCCCGGAAACAGAATCTTCATGAATGGATTAATGAGCTATTAAGAAAGTGGCTTGGTTATTGCACATGC TGGCGGCGTAATGACCTCCACCATGATGTTATCCAGCATGAAGGTCCTCACCAGAAGTCATACAAATCTTCTTAGGCTTC CAGAGTCGTGAGCAAAAAAAGCACACCTCTAAATAAATTAACTAGCCTCAGGTAGTTAACCACCGAAAATGAACCAAGGC AGTTCTAATACAAAACCACTTCCCTTCCCTGTTCAAAACCACAGTGCCCTATTATCTAAAAGATAAACTTCAAGCCAAGCT 35 TTTAGGTTGCCAGTATTTATGTAACAACAAGGCCCGTTGACACACATCTGTAACTCCTAGTACTGGGCCTCAGGGGCAGA

AGGATATCTGATATTGACTTCTGGCCAACACACAGCGTTTCTGCACATCTGTAGTTGCAAGCCTTTTGCACTARUTTTU GCCAGAGTCAGAGTTTGCAAGTGTTTGTGGACTGAATGCACGTGTTGGTGGTGATCTAGAAAGTCACCCTCCTTCTCAAG CTAGCAGCACTGGCTTCGGCCAGCTGCTCATTCAAGCCTCTTTGCAGAGTCATCACGGGGATGGGGGAGCAGUGCCCTT CCTAGARCACCAAGCCTGTGGTTGTTTATTCAGGACATTATTGAGGGCCAAGATGACAGATAACTCTATCACTTGGCCAA TTTTTCATTCAGGCRACTAGATTCCGTGGTACAARAGGCTCCCTGGGGRRCGAGGCCGGGRCAGGCGGGCTCCTGAGTCG GTCTGTGTACTCACAGGGAGGAGGGTGGCAAAGCCCTGGTCCTCTACGGGCTGGGGGAAGGGGAAGCTGTCGGCCCAG TGACTTTTTCCCCCTTTCTCTTTTCTTAGAAACCAGTCTCAATTTAAGATAATGAGTCTCCTCATTCACGTGTGCTCACT 10 AAAATGTGGCTGGACCGTGTGCCGGCACGAAACCAGGGATGTGAGGTCTAAGTTACATGCTCTGCCAGCCCCGGTGCCT TTTCCTTTCGGAAAGGAGACCCGGAGGTAAAACGAAGTTGCCAACTTTTGATGATGGTGTGCGCCGGGTGACTCTTTAAA 15 TTGCCCTTTTAGTTCCTAGAAAGCAGCACCGTAGTCTTGGCAGGTGGGCCATTGGTCACTCCGCTACCACTGTTACCATG TGAGAACTGGAGTTCAATTCCCAGCACATGGATGTATTTCCAGCACCTGGAAGGCAGGGAGCAGAGATCTTAAAGCTCCT GGCCAGACAGCCCAGCCTAATTAGTAATCAGTGAGAGACCCTGTCTCAAGAAAAAAAGATGGAACATCAAAGGTCAACCTC 20 GCTCTTGTCACCCCCACTAAGGCTTCAACTTCTTCTATTTCTTCATCTTGACCTCTGTACTTTGCATGCCTTTTCCAG ${\tt AAGTAGTCCAACCTCTTGGTGCTGCACCTGGACCCTGGACCCTGCACACACCACTGCTACCCAGCCCTGCAAACC}$ 25 TTCAGCCTAGCCTCTGGTTCTCCAACCAGCACAGGCCCAGTCTGGCTTCTATGTCCTAGAAATCTCCTTCATTCTCTCCA TTTCCCTCCTGAATCTACCACCTTCTTTCTCCCTTCTCCTGACCTCTAATGTCTTGGTCAAACGATTACAAGGAAGCCAA TGAAATTAGCAGTTTGGGGTACCTCAGAGTCAGCAGGGGAGGAGCTGGGATGAATTTCACATTTCCAGGCCTTTGCTTCC 30 $\tt GTGGTGGTGGTGGTGGTGTGTGTGTGTTTTTCTGCTTTTACAAAACTTTTCTAATTCTTATACAAAC$ GACAPATCTGCCTCATATAGGCAGAPAGATGACTTATGCCTATATAAGATATAPAGATGACTTTATGCCACTTATTAGCA ATAGTTACTGTCAAAAGTAATTCTATTTATACACCCTTATACATGGTATTGCTTTTGTTGGAGACTCTAAAATCCAGATT ATGTATTTAAAAAAAAATTCCCCAGTCCTTAAAAGGTGAAGAATGGACCCAGATAGAAGGTCACGGCACAAGTATGGAGT CGGAGTGTGGAGTCCTGCCAATGGTCTGGACAGAAGCATCCAGAGAGGGTCCAAGAAATGCCTCGCCTCCTAAGGAAC 35 ACTGGCAGCCCTGATGAGGTACCAGAGATTGCTAAGTGGAGGAATACAGGATCAGACCCATGGAGGGGCTTAAAGCGTGA

CTSTAGCA SCOCTCOGCTGAGGGGCTCCAGGTGGGCGCCCAAGGTGCTGCAGTGGGAGCCACATGAGAGGTGATGTCTTG GAGTCACCTCGGGTACCATTGTTTAGGGAGGTGGGGGATTTGTGGTGTGGAGACAGGCAGCCTCAAGGATGCTTTTCAACA ATGGTTGATGAGTTGGAACTAAAACAGGGGCCATCACACTGGCTCCCATAGCTCTGGGCTTGCCAGCTTCCACATCTGCC CCCCACCCCTGTCTGGCACCAGCTCAAGCTCTGTGATTCTACACATCCAAAAGAGGAAGAGTAGCCTACTGGGCATGCC 5 ACCTOTTCTGGACCATCAGGTGAGAGTGTGGCAAGCCCTAGGCTCCTGTCCAGGATGCAGGACGCCAGATAGGATGCTC TTGAGACAGGGTTTCTCTGTGTAGTCCTGGGTGTCCTGGAACTCACTTTGTAGACCAGGCTGGCCTCGAACTCAGAAATC 10 CTGGGATTAAAGGTGTGCGCACCACGCCCGGCCCTAACCCCCATTCTTAATGGTGATCCAGTGGTTGAAATTTCGGGCC ACACACATGTCCATTAGGGATTAGCTGCTGTCTTGAGCTACCTGGTAC&ATCTTTATCCCCTGGGGCCTGGGCTCCTG ATCCCTGACTCGGGCCCGATCAAGTCCAGTTCCTGGGCCCGATCAAGTCCAGTTCCTGGGCCCGAACAAGTCCAGTCCCT CTCAAGTTGTCTGCCACAGTCCCTAAGCCACCTCTGTAAGACAACTAAGATAATACTTCCCTCAAGCACGGAAAGTCCTG 15

Sequence ID No. 18: Human Beer Genomic Sequence (This gene has two exons, at positions 161-427 abd 3186-5219).

20

30

35

aacttetett tgggaggett ggaagaetgg ggtagaeeea gtgaagattg etggeetetg 540 ccagcactgg tcgaggaaca gtcttgcctg gaggtggggg aagaatggct cgctggtgca 600 gccttcaaat tcaggtgcag aggcatgagg caacagacgc tggtgagagc ccagggcagg 660 5 gaggacgctg gggtggtgag ggtatggcat cagggcatca gaacaggctc aggggctcag 720 aaaagaaaag gtttcaaaga atctcctcct gggaatatag gagccacgtc cagctgctgg 780 10 taccactggg aagggaacaa ggtaagggag ceteceatee acagaacage acetgtgggg 840 caccggacac tetatgetgg tggtggetgt ceccaceaca cagacecaca teatggaate 900 cccaggaggt gaacccccag ctcgaagggg aagaaacagg ttccaggcac tcagtaactt 960 15 ggtagtgaga agagctgagg tgtgaacctg gtttgatcca actgcaagat agccctggtg 1020 tgtggggggg tgtgggggac agatctccac aaagcagtgg ggaggaaggc cagagaggca 1080 20 cccctgcagt gtgcattgcc catggcctgc ccagggagct ggcacttgaa ggaatgggag 1140 ttttcggcac agttttagcc cctgacatgg gtgcagctga gtccaggccc tggaggggag 1200 agcagcatcc tctgtgcagg agtagggaca tctgtcctca gcagccaccc cagtcccaac 1260 25 cttgcctcat tccaggggag ggagaaggaa gaggaaccct gggttcctgg tcaggcctgc 1320 acagagaage ceaggtgaca gtgtgeatet ggetetataa ttggeaggaa teetgaggee 1380 30 atgggggcgt ctgaaatgac acttcagact aagagcttcc ctgtcctctg gccattatcc 1440 aggtggcaga gaagtccact gcccaggctc ctggacccca gccctccccg cctcacaacc 1500 tgttgggact atggggtgct aaaaagggca actgcatggg aggccagcca ggaccctccg 1560 35

tetteaaaat ggaggacaag ggegeeteee eecacagete eeettetagg caaggteage 1620 tgggctccag cgactgcctg aagggctgta aggaacccaa acacaaaatg tccaccttgc 1680 tggactccca cgagaggcca cagcccctga ggaagccaca tgctcaaaac aaagtcatga 1740 tctgcagagg aagtgcctgg cctaggggcg ctattctcga aaagccgcaa aatgcccct 1800 tccctgggca aatgcccccc tgaccacaca cacattccag ccctgcagag gtgaggatgc 1860 10 aaaccagccc acagaccaga aagcagcccc agacgatggc agtggccaca tctcccctgc 1920 tgtgcttgct cttcagagtg ggggtggggg gtggccttct ctgtcccctc tctggtttgg 1980 tcttaagact atttttcatt ctttcttgtc acattggaac tatccccatg aaacctttgg 2040 15 gggtggactg gtactcacac gacgaccagc tatttaaaaa gctcccaccc atctaagtcc 2100 accataggag acatggtcaa ggtgtgtgca ggggatcagg ccaggcctcg gagcccaatc 2160 20 tctgcctgcc cagggagtat caccatgagg cgcccattca gataacacag aacaagaaat 2220 gtgcccagca gagagccagg tcaatgtttg tggcagctga acctgtaggt tttgggtcag 2280 agctcagggc ccctatggta ggaaagtaac gacagtaaaa agcagccctc agctccatcc 2340 25 cccagcccag cctcccatgg atgctcgaac gcagagcctc cactcttgcc ggagccaaaa 2400 ggtgctggga ccccagggaa gtggagtccg gagatgcagc ccagcctttt gggcaagttc 2460 30 ttttctctgg ctgggcctca gtattctcat tgataatgag ggggttggac acactgcctt 2520 tgattccttt caagtctaat gaattcctgt cctgatcacc tccccttcag tccctcgcct 2580 ccacagcage tgccctgatt tattaccttc aattaacctc tactcctttc tccatcccct 2640

gtccacccct cccaagtggc tggaaaagga atttgggaga agccagagcc aggcagaagg 2700 tgtgctgagt acttaccctg cccaggccag ggaccctgcg gcacaagtgt ggcttaaatc 2760 ataagaagac cccagaagag aaatgataat aataatacat aacagccgac gctttcagct 2820 atatgtgcca aatggtattt tctgcattgc gtgtgtaatg gattaactcg caatgcttgg 2880 ggcggcccat tttgcagaca ggaagaagag agaggttaag gaacttgccc aagatgacac 2940 10 ctgcagtgag cgatggagcc ctggtgtttg aaccccagca gtcatttggc tccgagggga 3000 cagggtgcgc aggagagctt tccaccagct ctagagcatc tgggaccttc ctgcaataga 3060 15 tgttcagggg caaaagcctc tggagacagg cttggcaaaa gcagggctgg ggtggagaga 3120 gacgggccgg tccagggcag gggtggccag gcgggggcc accctcacgc gcgcctctct 3180 ccacagacgt gtccgagtac agctgccgcg agctgcactt cacccgctac gtgaccgatg 3240 20 ggccgtgccg cagcgccaag ccggtcaccg agctggtgtg ctccggccag tgcggcccgg 3300 cgcgcctgct gcccaacgcc atcggccgcg gcaagtggtg gcgacctagt gggcccgact 3360 teegetgeat eccegacege tacegegege agegegtgea getgetgtgt eccggtggtg 3420 25 aggcgccgcg cgcgcgcaag gtgcgcctgg tggcctcgtg caagtgcaag cgcctcaccc 3480 gcttccacaa ccagtcggag ctcaaggact tcgggaccga ggccgctcgg ccgcagaagg 3540 30 gccggaagcc gcggccccgc gcccggagcg ccaaagccaa ccaggccgag ctggagaacg 3600 cctactagag cccgccgcg cccctcccca ccggcggcg ccccggccct gaacccgcgc 3660 cccacatttc tgtcctctgc gcgtggtttg attgtttata tttcattgta aatgcctgca 3720 35

acccagggca gggggctgag accttccagg ccctgaggaa tcccgggcgc cggcaaggcc 3780 cccctcagcc cgccagctga ggggtcccac ggggcagggg agggaattga gagtcacaga 3840 cactgagcca cgcagccccg cctctggggc cgcctacctt tgctggtccc acttcagagg 3900 aggcagaaat ggaagcattt tcaccgccct ggggttttaa gggagcggtg tgggagtggg 3960 aaagtccagg gactggttaa gaaagttgga taagattccc ccttgcacct cgctgcccat 4020 10 cagaaagcct gaggcgtgcc cagagcacaa gactgggggc aactgtagat gtggtttcta 4080 gtcctggctc tgccactaac ttgctgtgta accttgaact acacaattct ccttcgggac 4140 ctcaatttcc actttgtaaa atgagggtgg aggtgggaat aggatctcga ggagactatt 4200 ggcatatgat tccaaggact ccagtgcctt ttgaatgggc agaggtgaga gagagagaga 4260 gaaagagaga gaatgaatgc agttgcattg attcagtgcc aaggtcactt ccagaattca 4320 20 gagttgtgat gctctcttct gacagccaaa gatgaaaaac aaacagaaaa aaaaaagtaa 4380 agagtctatt tatggctgac atatttacgg ctgacaaact cctggaagaa gctatgctgc 4440 ttcccagcct ggcttccccg gatgtttggc tacctccacc cctccatctc aaagaaataa 4500 25 catcatccat tggggtagaa aaggagaggg tccgagggtg gtgggaggga tagaaatcac 4560 atcogococa acttoccaaa gagoagoato ootooooga oocatagooa tgttttaaag 4620 30 tcaccttccg aagagaagtg aaaggttcaa ggacactggc cttgcaggcc cgagggagca 4680 gccatcacaa actcacagac cagcacatcc cttttgagac accgccttct gcccaccact 4740 cacggacaca tttctgccta gaaaacagct tcttactgct cttacatgtg atggcatatc 4800 35

ttacactaaa agaatattat tgggggaaaa actacaagtg ctgtacatat gctgagaaac 4860 tgcagagcat aatagctgcc acccaaaaat ctttttgaaa atcatttcca gacaacctct 4920 tactttctgt gtagttttta attgttaaaa aaaaaaagtt ttaaacagaa gcacatgaca 4980 tatgaaagcc tgcaggactg gtcgtttttt tggcaattct tccacgtggg acttgtccac 5040 aagaatgaaa gtagtggttt ttaaagagtt aagttacata tttattttct cacttaagtt 5100 10 atttatgcaa aagtttttct tgtagagaat gacaatgtta atattgcttt atgaattaac 5160 agtotgttot tocagagtor agagacattg ttaataaaga caatgaatca tgaccgaaag 5220 gatgtggtct cattttgtca accacacatg acgtcatttc tgtcaaagtt gacacccttc 5280 15 tettggteae tagageteea acettggaea caeetttgae tgetetetgg tggeeettgt 5340 ggcaattatg tetteetttg aaaagteatg tttateeett eettteeaaa eecagaeege 5400 20 atticticac ccagggcatg gtaataacci cagccitgta tccitttagc agcciccct 5460 ccatgctggc ttccaaaatg ctgttctcat tgtatcactc ccctgctcaa aagccttcca 5520 tagctccccc ttgcccagga tcaagtgcag tttccctatc tgacatggga ggccttctct 5580 25 gcttgactcc cacctcccac tccaccaagc ttcctactga ctccaaatgg tcatgcagat 5640 ccctgcttcc ttagtttgcc atccacactt agcaccccca ataactaatc ctctttcttt 5700 30 aggattcaca ttacttgtca tetetteece taacetteea gagatgttee aateteecat 5760 gatecetete teetetgagg trecageece trrigterae accaetaetr tggrteetaa 5820 ttctgttttc catttgacag tcattcatgg aggaccagcc tggccaagtc ctgcttagta 5880

ctggcataga caacacaaag ccaagtacaa ttcaggacca gctcacagga aacttcatct 5940 tcttcgaagt gtggatttga tgcctcctgg gtagaaatgt aggatcttca aaagtgggcc 6000 agectectge actteteta aagtetegee teeceaaggt gtettaatag tgetggatge 6060 tagctgagtt agcatcttca gatgaagagt aaccctaaag ttactcttca gttgccctaa 6120 ggtgggatgg tcaactggaa agctttaaat taagtccagc ctaccttggg ggaacccacc 6180 10 cccacaaaga aagctgaggt ccctcctgat gacttgtcag tttaactacc aataacccac 6240 ttgaattaat catcatcatc aagtctttga taggtgtgag tgggtatcag tggccggtcc 6300 cttcctgggg ctccagcccc cgaggaggcc tcagtgagcc cctgcagaaa atccatgcat 6360 15 catgagtgtc tcagggccca gaatatgaga gcaggtagga aacagagaca tcttccatcc 6420 ctgagaggca gtgcggtcca gtgggtgggg acacgggctc tgggtcaggt ttgtgttgtt 6480 20 tgtttgtttg ttttgagaca gagtctcgct ctattgccca ggctggagtg cagtgtcaca 6540 atctcggctt actgcaactt ctgccttccc ggattcaagt gattctcctg cctcagcctc 6600 cagagtagct gggattacag gtgcgtgcca ccacgcctgg ctaatttttg tatttttgat 6660 25 agagacgggg tttcaccatg ttggccaggc tagtctcgaa ctcttgacct caagtgatct 6720 geetgeeteg geeteecaaa gtgetgggat tacaggegtg ageeaceaca eccageecca 6780 30 ggttggtgtt tgaatctgag gagactgaag caccaagggg ttaaatgttt tgcccacagc 6840 catacttggg ctcagttcct tgccctaccc ctcacttgag ctgcttagaa cctggtgggc 6900 acatgggcaa taaccaggtc acactgtttt gtaccaagtg ttatgggaat ccaagatagg 6960

agtaatttgc tctgtggagg ggatgaggga tagtggttag ggaaagcttc acaaagtggg 7020 tgttgcttag agattttcca ggtggagaag ggggcttcta ggcagaaggc atagcccaag 7080 caaagactgc aagtgcatgg ctgctcatgg gtagaagaga atccaccatt cctcaacatg 7140 taccgagtcc ttgccatgtg caaggcaaca tgggggtacc aggaattcca agcaatgtcc 7200 aaacctaggg tetgetttet gggacetgaa gatacaggat ggateageee aggetgeaat 7260 10 cccattacca cgagggggaa aaaaacctga aggctaaatt gtaggtcggg ttagaggtta 7320 tttatggaaa gttatattct acctacatgg ggtctataag cctggcgcca atcagaaaag 7380 gaacaaacaa cagacctagc tgggagggc agcattttgt tgtagggggc ggggcacatg 7440 ttctgggggt acagccagac tcagggcttg tattaatagt ctgagagtaa gacagacaga 7500 gggatagaag gaaataggtc cctttctctc tctctctc tctctctc actctctc 7560 20 teteteacae acaeacaeag acaeacaea aegetetgta ggggtetaet tatgeteeaa 7620 gtacaaatca ggccacattt acacaaggag gtaaaggaaa agaacgttgg aggagccaca 7680 ggaccccaaa attccctgtt ttccttgaat caggcaggac ttacgcagct gggagggtgg 7740 25 agagcctgca gaagccacct gcgagtaagc caagttcaga gtcacagaca ccaaaagctg 7800 gtgccatgtc ccacacccgc ccacctccca cctgctcctt gacacagccc tgtgctccac 7860 30 aacceggete ceagateatt gattataget etggggeetg cacegteett eetgeeacat 7920 ccccacccca ttcttggaac ctgccctctg tcttctccct tgtccaaggg caggcaaggg 7980 ctcagctatt gggcagcttt gaccaacagc tgaggctcct tttgtggctg gagatgcagg 8040 35

aggcagggga atattcctct tagtcaatgc gaccatgtgc ctggtttgcc cagggtggtc 8100 tcgtttacac ctgtaggcca agcgtaatta ttaacagctc ccacttctac tctaaaaaat 8160 gacccaatct gggcagtaaa ttatatggtg cccatgctat taagagctgc aacttgctgg 8220 gegtggtgge teacacetgt aateecagta etttgggaeg teaaggeggg tggateacet 8280 gaggtcacga gttagagact ggcctggcca gcatggcaaa accccatctt tactaaaaat 8340 10 acaaaaatta gcaaggcatg gtggcatgca cctgtaatcc caggtactcg ggaggctgag 8400 acaggagaat ggcttgaacc caggaggcag aggttgcagt gagccaagat tgtgccactg 8460 ccctccagcc ctggcaacag agcaagactt catctcaaaa gaaaaaggat actgtcaatc 8520 actgcaggaa gaacccaggt aatgaatgag gagaagagag gggctgagtc accatagtgg 8580 cagcaccgac tectgeagga aaggegagae aetgggteat gggtaetgaa gggtgeeetg 8640 20 aatgacgttc tgctttagag accgaacctg agccctgaaa gtgcatgcct gttcatgggt 8700 gagagactaa attcatcatt ccttggcagg tactgaatcc tttcttacgg ctgccctcca 8760 atgcccaatt tccctacaat tgtctggggt gcctaagctt ctgcccacca agagggccag 8820 25 agctggcagc gagcagctgc aggtaggaga gataggtacc cataagggag gtgggaaaga 8880 gagatggaag gagaggggtg cagagcacac acctcccctg cctgacaact tcctgagggc 8940 30 tggtcatgcc agcagattta aggcggaggc aggggagatg gggcgggaga ggaagtgaaa 9000 aaggagaggg tggggatgga gaggaagaga gggtgatcat tcattcattc cattgctact 9060 gactggatgc cagctgtgag ccaggcacca ccctagctct gggcatgtgg ttgtaatctt 9120 35

ggagcctcat ggagctcaca gggagtgctg gcaaggagat ggataatgga cggataacaa 9180

ataaacattt agtacaatgt ccgggaatgg aaagttctcg aaagaaaaat aaagctggtg 9240

agcatataga cagccctgaa ggcggccagg ccaggcattt ctgaggaggt ggcatttgag 9300

c

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

CLAIMS

We claim:

- 1. An isolated nucleic acid molecule selected from the group consisting of:
- 5 (a) an isolated nucleic acid molecule comprising sequence ID Nos., 1, 5, 9, 11, 13, or, 15, or complementary sequence thereof;
 - (b) an isolated nucleic acid molecule that specifically hybridizes to the nucleic acid molecule of (a) under conditions of high stringency, and
- (c) an isolated nucleic acid that encodes a TGF-beta binding-protein according to (a) or (b)
 - 2. The isolated nucleic acid molecule according to claim 1 wherein said nucleic acid molecule encodes a protein comprising the protein of Sequence ID NO. 2.
- 3. The isolated nucleic acid molecule according to claim 1 wherein said nucleic acid molecule encodes a protein comprising the protein of Sequence ID NO. 6.
 - 4. The isolated nucleic acid molecule according to claim 1 wherein said nucleic acid molecule encodes a protein comprising the protein of Sequence ID NO. 10.
- 5. The isolated nucleic acid molecule according to claim 1 wherein said nucleic acid molecule encodes a protein comprising the protein of Sequence ID NO. 12.
- 6. The isolated nucleic acid molecule according to claim 1 wherein said nucleic acid molecule encodes a protein comprising the protein of Sequence ID NO. 14.
 - 7. The isolated nucleic acid molecule according to claim 1 wherein said nucleic acid molecule encodes a protein comprising the protein of Sequence ID NO. 16.

5

- 8. An expression vector, comprising a promoter operably linked to a nucleic acid molecule according to any one of claims 1 to 7.
- 9. The expression vector according to claim 8 wherein said promoter is selected from the group consisting of CMV I-E promoter, SV40 early promoter and MuLV LTR.
- 10. The expression vector according to claim 8 wherein said promoter is a tissue-specific promoter.
- 11. A method of producing a TGF-beta binding protein, comprising, culturing a cell which contains a vector according to claim 8 under conditions and for a time sufficient to produce said protein.
 - 12. The method according to claim 11, further comprising the step of purifying said protein.
 - 13. A viral vector capable of directing the expression of a nucleic acid molecule according to any one of claims 1 to 7.
- 15 14. The viral vector according to claim 13 wherein said vector is selected from the group consisting of herpes simplex viral vectors, adenovirus-associated viral vectors and retroviral vectors.
 - 15. A host cell carrying a vector according to any one of claims 8 to 14.
- from the group consisting of a human cell, dog cell, monkey cell, rat cell and mouse cell.
 - 17. An isolated protein, comprising a TGF-beta binding-protein encoded by the nucleic acid molecule according to any one of claims 1 to 7.
- 25 An antibody which specifically binds to the protein according to claim 17.

- 19. The antibody according to claim 18 wherein said antibody is a monoclonal antibody.
- 20. The antibody according to claim 19 wherein said monoclonal antibody is a murine or human antibody.
- 5 21. The antibody according to claim 18 wherein said antibody is selected from the group consisting of F(ab')₂, F(ab)₂, Fab', Fab, and Fv.
 - A hybridoma which produces an antibody according to claim 19.
- 23. A fusion protein, comprising a first polypeptide segment comprising a TGF-beta binding-protein encoded by the nucleic acid molecule according to any one of claims 1 to 7, or a portion thereof of at least 10 amino acids in length, and a second polypeptide segment comprising a non-TGF-beta binding-protein.
 - 24. The fusion protein according to claim 23 wherein said first polypeptide segment is at least 20 amino acids in length.
- 25. The fusion protein according to claim 23 wherein said first polypeptide segment is at least 50 amino acids in length.
 - 26. The fusion protein according to claim 23 wherein said second polypeptide comprises multiple anionic amino acid residues.
- 27. An isolated oligonucleotide which hybridizes to a nucleic acid molecule according to Sequence ID NOs. 1, 3, 5, 7, 9, 11, 13, or 15, or the complement thereto, under conditions of high stringency.
 - 28. The isolated oligonucleotide according to claim 27 wherein said oligonucleotide is at least 20 nucleotides in length.
 - 29. The isolated oligonucleotide according to claim 27 wherein said oligonucleotide is at least 30 nucleotides in length.
- 25 The isolated oligonucleotide according to claim 27 wherein said

oligonucleotide is at least 50 nucleotides in length.

- 31. The isolated oligonucleotide according to claim 27 wherein said oligonucleotide is between 50 to 100 nucleotides in length.
- 32. A pair of primers which specifically amplifies all or a portion of a nucleic acid molecule according to any one of claims 1 to 7.
 - A ribozyme which cleaves RNA encoding a protein according to claim 17.
 - 34. The ribozyme according to claim 33 wherein said protein comprises the protein of Sequence ID NO. 2.
- The ribozyme according to claim 33 wherein said protein comprises the protein of Sequence ID NO. 6.
 - 36. The ribozyme according to claim 33 wherein said RNA encodes a protein comprising the protein of Sequence ID NO. 10.
- 37. The ribozyme according to claim 33 wherein said RNA encodes a protein comprising the protein of Sequence ID NO. 12.
 - 38. The ribozyme according to claim 33 wherein said RNA encodes a protein comprising the protein of Sequence ID NO. 14.
 - 39. The ribozyme according to claim 33 wherein said RNA encodes a protein comprising the protein of Sequence ID NO. 16.
- 20 40. The ribozyme according to claim 33 wherein said ribozyme is composed of ribonucleic acids.
 - 41. The ribozyme according to claim 40 wherein one or more of said ribonucleic acids are 2'-O-methyl ribonucleic acids.
 - 42. The ribozyme according to claim 33 wherein said ribozyme is

composed of a mixture of deoxyribonucleic acids and ribonucleic acids.

- 43. The ribozyme according to claim 33 wherein said ribozyme is composed of nucleic acids having phosphothioate linkages.
- 44. A nucleic acid molecule comprising a nucleic acid sequence which encodes a ribozyme according to claim 33.
 - The nucleic acid molecule of claim 44, wherein the nucleic acid is DNA or cDNA.
 - 46. The nucleic acid molecule of claim 44, under the control of a promoter to transcribe the nucleic acid.
- 10 47. A host cell comprising the ribozyme of claim 33.
 - 48. A vector, comprising the nucleic acid molecule of claim 44.
 - 49. The vector of claim 54, wherein the vector is a plasmid, a virus, retrotransposon or a cosmid.
- 50. The vector of claim 49 wherein said virus is selected from the group consisting of retroviruses, adenoviruses, and adeno-associated viruses.
 - 51. A host cell containing the vector according to any one of claims 48 to 50.
 - 52. The host cell according to claim 51 wherein said host cell is stably transformed with said vector.
- The host cell according to claim 51 wherein the host cell is a human cell.
 - 54. A method for producing a ribozyme, comprising providing DNA encoding the ribozyme according to claim 33 under the transcriptional control of a promoter, and transcribing the DNA to produce the ribozyme.

- 55. The method of claim 54 wherein the ribozyme is produced in vitro.
- 56. The method of claim 54, further comprising purifying the ribozyme.
- 57. A method for increasing bone mineralization, comprising introducing into a warm-blooded animal an effective amount of the ribozyme according to any one of claims 33 to 43.
- 58. A method of increasing bone mineralization, comprising introducing into a patient an effective amount of the nucleic acid molecule of claim 44, under conditions favoring transcription of the nucleic acid molecule to produce a ribozyme.
 - 59. A pharmaceutical composition, comprising the ribozyme according to any one of claims 33 to 43, and a pharmaceutically acceptable carrier or diluent.
- 15 60. A pair of primers capable of specifically amplifying all or a portion of a nucleic acid molecule according to any one claims 1 to 7.
- 61. A method for detecting a nucleic acid molecule which encodes a TGF-beta binding protein, comprising incubating an oligonucleotide according to any one of claims 27 to 31 under conditions of high stringency, and detecting hybridization of said oligonucleotide.
 - 62. The method according to claim 61 wherein said oligonucleotide is labeled.
 - 63. The method according to claim 61 wherein said oligonucleotide is bound to a solid support.
- 25 64. A method for detecting a TGF-beta binding protein, comprising incubating an antibody according to any one of claims 18 to 21 under conditions and for a time sufficient to permit said antibody to bind to a TGF-beta binding protein, and

detecting said binding.

- 65. The method according to claim 64 wherein said antibody is bound to a solid support.
- 66. The method according to claim 64 wherein said antibody is labeled.
 - 67. The method according to claim 66 wherein said antibody is labeled with a marker selected from the group consisting of enzymes, fluorescent proteins, and radioisotopes.
- 68. A transgenic animal whose germ cells and somatic cells contain a nucleic acid molecule encoding a TGF-beta binding-protein according to claim 1 which is operably linked to a promoter effective for the expression of said gene, said gene being introduced into said animal, or an ancestor of said animal, at an embryonic stage, with the proviso that said animal is not a human.
- 69. The transgenic animal according to claim 68 wherein TGF-beta binding-protein is expressed from a vector according to any one of claims 8 to 10.
 - 70. A transgenic knockout animal, comprising an animal whose germ cells and somatic cells comprise a disruption of at least one allele of an endogenous nucleic acid molecule which hybridizes to the nucleic acid molecule according to claim 1, wherein said disruption prevents transcription of messenger RNA from said allele as compared to an animal without said disruption, with the proviso that said animal is not a human.
 - 71. The transgenic animal according to claim 70 wherein said disruption is a nucleic acid deletion, substitution, or, insertion.
- 72. The transgenic animal according to claim 68 or 70 wherein the animal is selected from the group consisting of a mouse, a rat and a dog.

20

- 73. A method for determining whether a candidate molecule is capable of increasing bone mineral content, comprising:
- (a) mixing one or more candidate molecules with TGF-beta-binding-protein encoded by the nucleic acid molecule according to any one of claims 1 to 7 and a selected member of the TGF-beta family of proteins;
- (b) determining whether the candidate molecule alters the signaling of the TGF-beta family member, or alters the binding of the TGF-beta binding-protein to the TGF-beta family member.
- 74. The method according to claim 73 wherein said member of the TGF-beta family of proteins is BMP6.
 - 75. A method for determining whether a candidate molecule is capable of increasing bone mineral content, comprising: determining whether a candidate molecule inhibits the binding of TGF-beta binding-protein to bone, or an analogue thereof.
- The method according to claim 75 wherein said analogue of bone is hydroxyapatite.
 - 77. A kit for detection of TGF-beta binding-protein gene expression, comprising a container that comprises a nucleic acid molecule, wherein said nucleic acid molecule is selected from the group consisting of (a) a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO: 1, 5, 7, 9, 11, 13, or 15; (b) a nucleic acid molecule comprising the complement of the nucleotide sequence of (a); (c) a nucleic acid molecule that is a fragment of (a) or (b) of at least 20 nucleotides in length.
- A kit for detection of TGF-beta binding-protein, comprising a container that comprises an antibody according to any one of claims 18 to 21.
- 79. An antisense oligonucleotide, comprising a nucleic acid molecule which hybridizes to a nucleic acid molecule according to Sequence ID NOs. 1, 3, 5, 7, 9, 11, 13, or 15, or the complement thereto, and wherein said oligonucleotide inhibits the expression of TGF-beta binding protein according to

20

claim 17.

- The oligonucleotide according to claim 79 wherein said oligonucleotide is 15 nucleotides in length.
- The oligonucleotide according to claim 79 wherein said oligonucleotide is 20 nucleotides in length.
 - 82. The oligonucleotide according to claim 79 wherein said oligonucleotide is 50 nucleotides in length.
 - The oligonucleotide according to claim 79, wherein said oligonucleotide is comprised of one or more nucleic acid analogs.
- The oligonucleotide according to claim 79, wherein said oligonucleotide is comprised of one or more ribonucleic acids.
 - The oligonucleotide according to claim 79, wherein said oligonucleotide is comprised of one or more deoxyribonucleic acids.
- The oligonucleotide according to claim 79 wherein said oligonucleotide sequence comrpises one or more modified covalent linkages.
 - 87. The oligonucleotide according to claim 86 wherein said modified covalent linkage is selected from the group consisting of a phosphorothioate linkage, a phosphotriester linkage, a methyl phosphonate linkage, a methylene(methylimino) linkage, a morpholino linkage, an amide linkage, a polyamide linkage, a short chain alkyl intersugar linkage, a cycloalkyl intersugar linkage, a short chain heteroatomic intersugar linkage and a heterocyclic intersugar linkage.

Common Cysteine Backbone

<pre>1 human_gremlin.pro</pre>				50	
human_cerberus.pro	-	J LLPLGKTTRH	H QDGRQNQSSL	. SPVLLPRNQR	ELPTGNHEEA
human_dan.pro human beer.pro				. ~~~~~~	
numan_beer.pro					
	51				
human_gremlin.pro	- -	M	S CDTAVTUCAT	7 7 7 7 0m	100 AEGKKKGSQG
human_cerberus.pro	EEKPDLFVA	PHLVAT.SPA	GEGOROPEKM	LSRFGRFWKK	AEGKKKGSQG
human_dan.pro	~~~~~		· crookokokok	LSRFGRFWKK	PEREMHPSRD
human_beer.pro	~~~~~~			MQLPLA	LCLUCLLUUT
				IL ILL	DCDVCDDVAI
human_gremlin.pro	101				150
human_cerberus.pro	SDSEDEDDGT	HNDSEQTQSP	QQPGSRNRGR	GQGRGTAMPG	EEVLESSQEA
human dan.pro	opped feed.	CSTICLID.G	MKMEKSPLRE	FAKKEWUUEM	EDVTDACOGU
human_beer.pro	AFRVVEGOGW	OAFKNDATET	TDEL CHYPER	MLRVLVGAVL PPELENNKTM	PAMLLAAPPP
		•	TPELGETPEP	PPELENNKTM	NRAENGGRPP
.	151	Ψ	\mathbf{V}	Ψ	V 200
human_gremlin.pro	LHVTERKYLK	RDWCKTQPLK	QTIHEEGCNS	RTIINRF.CY	COCNCENTER
human_cerberus.pro	ILPIKSHEVH	WETCDTUDEC	OET ELLE		- 2 - 1 - 1 - 1 - 1 - 1 - 1 - 1 - 1
human dan	711111	WEICKIVPFS	OTTTHEGCEK	VVVQNNL.CF	GKCGSVHFP.
human_dan.pro	TITIES FOR	SAMCEAKULL	OIVGHSGCEA	VVVQNNL.CF KSIQNRA.CL	COCCOUNT
human_dan.pro human_beer.pro	TITIES FOR	SAMCEAKULL	OIVGHSGCEA	KCIONDA CI	COCCOUNT
	HHPFETKDVS	SAMCEAKULL	OIVGHSGCEA	VVVQNNL.CF KSIQNRA.CL AKPVTELVCS	COCCOUNT
human_beer.pro	HHPFETKDVS	EYSCRELHFT	QIVGHSGCEA RYVTDGPCRS	KSIQNRA.CL AKPVTELVCS	GQCFSYSVPN GQCGPARLLP
human_beer.pro human_gremlin.pro	HHPFETKDVS 201 HIRKEEGSFQ	EYSCRELHFT SCSFCKP	QIVGHSGCEA RYVTDGPCRS	KSIQNRA.CL AKPVTELVCS V NCPELOPPTK	GQCFSYSVPN GQCGPARLLP 250
human_beer.pro	HHPFETKDVS 201 HIRKEEGSFQGAAQHSHT	EYSCRELHFT SCSFCKP SCSHCLP	QIVGHSGCEA RYVTDGPCRS KKFTTMMVTL AKFTTMHLDL	KSIQNRA.CL AKPVTELVCS V NCPELQPPTK	GQCFSYSVPN GQCGPARLLP 250 K.KRVTRVKQ
human_beer.pro human_gremlin.pro human_cerberus.pro	HHPFETKDVS 201 HIRKEEGSFQGAAQHSHT TFPQSTESLV	EYSCRELHFT SCSFCKP SCSHCLP HCDSCMP	QIVGHSGCEA RYVTDGPCRS KKFTTMMVTL AKFTTMHLPL AOSMWEIVTL	KSIQNRA.CL AKPVTELVCS V NCPELQPPTK NCTELSSVIK	GQCFSYSVPN GQCGPARLLP 250 K.KRVTRVKQ VVMLVEE
human_beer.pro human_gremlin.pro human_cerberus.pro human_dan.pro	HHPFETKDVS 201 HIRKEEGSFQGAAQHSHT TFPQSTESLV	EYSCRELHFT SCSFCKP SCSHCLP HCDSCMP	QIVGHSGCEA RYVTDGPCRS KKFTTMMVTL AKFTTMHLPL AOSMWEIVTL	KSIQNRA.CL AKPVTELVCS V NCPELQPPTK	GQCFSYSVPN GQCGPARLLP 250 K.KRVTRVKQ VVMLVEE
human_beer.pro human_gremlin.pro human_cerberus.pro human_dan.pro human_beer.pro	HHPFETKDVS 201 HIRKEEGSFQGAAQHSHT TFPQSTESLV NAIGRGKWWR	EYSCRELHFT SCSFCKP SCSHCLP HCDSCMP PSGPDFRCIP	QIVGHSGCEA RYVTDGPCRS KKFTTMMVTL AKFTTMHLPL AOSMWEIVTL	KSIQNRA.CL AKPVTELVCS V NCPELQPPTK NCTELSSVIK	GQCFSYSVPN GQCGPARLLP 250 K.KRVTRVKQ VVMLVEE VDKLVEKILH RKVRLVAS
human_beer.pro human_gremlin.pro human_cerberus.pro human_dan.pro human_beer.pro human_beer.pro	HHPFETKDVS 201 HIRKEEGSFQGAAQHSHT TFPQSTESLV NAIGRGKWWR VSV CRC.ISIDLD	SCSFCKP SCSHCLP HCDSCMP PSGPDFRCIP	QIVGHSGCEA RYVTDGPCRS KKFTTMMVTL AKFTTMHLPL AQSMWEIVTL DRYRAQRVQL	KSIQNRA.CL AKPVTELVCS V NCPELQPPTK NCTELSSVIK ECPGHEEVPR LCPGGEAPRA	GQCFSYSVPN GQCGPARLLP 250 K.KRVTRVKQ VVMLVEE VDKLVEKILH RKVRLVAS
human_beer.pro human_gremlin.pro human_cerberus.pro human_dan.pro human_beer.pro human_beer.pro human_gremlin.pro human_cerberus.pro	HHPFETKDVS 201 HIRKEEGSFQGAAQHSHT TFPQSTESLV NAIGRGKWWR VSV CRC.ISIDLD CQCKVKTEHE	SCSFCKP SCSHCLP HCDSCMP PSGPDFRCIP	QIVGHSGCEA RYVTDGPCRS KKFTTMMVTL AKFTTMHLPL AQSMWEIVTL DRYRAQRVQL	KSIQNRA.CL AKPVTELVCS V NCPELQPPTK NCTELSSVIK ECPGHEEVPR LCPGGEAPRA	GQCFSYSVPN GQCGPARLLP 250 K.KRVTRVKQ VVMLVEE VDKLVEKILH RKVRLVAS
human_beer.pro human_gremlin.pro human_cerberus.pro human_dan.pro human_beer.pro human_gremlin.pro human_cerberus.pro human_dan.pro	HHPFETKDVS 201 HIRKEEGSFQGAAQHSHT TFPQSTESLV NAIGRGKWWR CRC.ISIDLD CQCKVKTEHE CSCQACGKEP	SCSFCKP SCSHCLP HCDSCMP PSGPDFRCIP	QIVGHSGCEA RYVTDGPCRS KKFTTMMVTL AKFTTMHLPL AQSMWEIVTL DRYRAQRVQL DSFIPGVSA- GEDGPGSOPG	KSIQNRA.CL AKPVTELVCS V NCPELQPPTK NCTELSSVIK ECPGHEEVPR LCPGGEAPRA	GQCFSYSVPN GQCGPARLLP 250 K.KRVTRVKQ VVMLVEE VDKLVEKILH RKVRLVAS
human_beer.pro human_gremlin.pro human_cerberus.pro human_dan.pro human_beer.pro human_beer.pro human_gremlin.pro human_cerberus.pro	HHPFETKDVS 201 HIRKEEGSFQGAAQHSHT TFPQSTESLV NAIGRGKWWR CRC.ISIDLD CQCKVKTEHE CSCQACGKEP	SCSFCKP SCSHCLP HCDSCMP PSGPDFRCIP	QIVGHSGCEA RYVTDGPCRS KKFTTMMVTL AKFTTMHLPL AQSMWEIVTL DRYRAQRVQL DSFIPGVSA- GEDGPGSOPG	KSIQNRA.CL AKPVTELVCS V NCPELQPPTK NCTELSSVIK ECPGHEEVPR LCPGGEAPRA	GQCFSYSVPN GQCGPARLLP 250 K.KRVTRVKQ VVMLVEE VDKLVEKILH RKVRLVAS
human_beer.pro human_gremlin.pro human_cerberus.pro human_dan.pro human_beer.pro human_gremlin.pro human_cerberus.pro human_dan.pro	HHPFETKDVS 201 HIRKEEGSFQGAAQHSHT TFPQSTESLV NAIGRGKWWR CRC.ISIDLD CQCKVKTEHE CSCQACGKEP CKCKRLTRFH	SCSFCKP SCSHCLP HCDSCMP PSGPDFRCIP DGHILHAGSQ SHEGLSVYVQ NQSELKDFGT	QIVGHSGCEA RYVTDGPCRS KKFTTMMVTL AKFTTMHLPL AQSMWEIVTL DRYRAQRVQL DSFIPGVSA- GEDGPGSOPG	KSIQNRA.CL AKPVTELVCS V NCPELQPPTK NCTELSSVIK ECPGHEEVPR LCPGGEAPRA	GQCFSYSVPN GQCGPARLLP 250 K.KRVTRVKQ VVMLVEE VDKLVEKILH RKVRLVAS
human_beer.pro human_gremlin.pro human_cerberus.pro human_dan.pro human_beer.pro human_gremlin.pro human_cerberus.pro human_dan.pro human_dan.pro human_beer.pro	HHPFETKDVS 201 HIRKEEGSFQGAAQHSHT TFPQSTESLV NAIGRGKWWR CRC.ISIDLD CQCKVKTEHE CSCQACGKEP CKCKRLTRFH	SCSFCKP SCSHCLP HCDSCMP PSGPDFRCIP DGHILHAGSQ SHEGLSVYVQ NQSELKDFGT	QIVGHSGCEA RYVTDGPCRS KKFTTMMVTL AKFTTMHLPL AQSMWEIVTL DRYRAQRVQL DSFIPGVSA- GEDGPGSOPG	KSIQNRA.CL AKPVTELVCS V NCPELQPPTK NCTELSSVIK ECPGHEEVPR LCPGGEAPRA	GQCFSYSVPN GQCGPARLLP 250 K.KRVTRVKQ VVMLVEE VDKLVEKILH RKVRLVAS
human_gremlin.pro human_cerberus.pro human_dan.pro human_beer.pro human_gremlin.pro human_cerberus.pro human_dan.pro human_den.pro human_beer.pro	HHPFETKDVS 201 HIRKEEGSFQGAAQHSHT TFPQSTESLV NAIGRGKWWR CRC.ISIDLD CQCKVKTEHE CSCQACGKEP CKCKRLTRFH	SCSFCKP SCSHCLP HCDSCMP PSGPDFRCIP DGHILHAGSQ SHEGLSVYVQ NQSELKDFGT	QIVGHSGCEA RYVTDGPCRS KKFTTMMVTL AKFTTMHLPL AQSMWEIVTL DRYRAQRVQL DSFIPGVSA- GEDGPGSOPG	KSIQNRA.CL AKPVTELVCS V NCPELQPPTK NCTELSSVIK ECPGHEEVPR LCPGGEAPRA	GQCFSYSVPN GQCGPARLLP 250 K.KRVTRVKQ VVMLVEE VDKLVEKILH RKVRLVAS
human_gremlin.pro human_cerberus.pro human_dan.pro human_beer.pro human_gremlin.pro human_cerberus.pro human_dan.pro human_deer.pro human_dan.pro human_beer.pro human_beer.pro	HHPFETKDVS 201 HIRKEEGSFQGAAQHSHT TFPQSTESLV NAIGRGKWWR CRC.ISIDLD CQCKVKTEHE CSCQACGKEP CKCKRLTRFH 301	SCSFCKP SCSHCLP HCDSCMP PSGPDFRCIP DGHILHAGSQ SHEGLSVYVQ NQSELKDFGT	QIVGHSGCEA RYVTDGPCRS KKFTTMMVTL AKFTTMHLPL AQSMWEIVTL DRYRAQRVQL DSFIPGVSA- GEDGPGSOPG	KSIQNRA.CL AKPVTELVCS V NCPELQPPTK NCTELSSVIK ECPGHEEVPR LCPGGEAPRA	GQCFSYSVPN GQCGPARLLP 250 K.KRVTRVKQ VVMLVEE VDKLVEKILH RKVRLVAS
human_gremlin.pro human_cerberus.pro human_dan.pro human_beer.pro human_gremlin.pro human_cerberus.pro human_dan.pro human_den.pro human_beer.pro	HHPFETKDVS 201 HIRKEEGSFQGAAQHSHT TFPQSTESLV NAIGRGKWWR CRC.ISIDLD CQCKVKTEHE CSCQACGKEP CKCKRLTRFH	SAWCEARNIT EYSCRELHFT SCSFCKP SCSHCLP HCDSCMP PSGPDFRCIP DGHILHAGSQ SHEGLSVYVQ NQSELKDFGT 314 GAED	QIVGHSGCEA RYVTDGPCRS KKFTTMMVTL AKFTTMHLPL AQSMWEIVTL DRYRAQRVQL DSFIPGVSA- GEDGPGSOPG	KSIQNRA.CL AKPVTELVCS V NCPELQPPTK NCTELSSVIK ECPGHEEVPR LCPGGEAPRA	GQCFSYSVPN GQCGPARLLP 250 K.KRVTRVKQ VVMLVEE VDKLVEKILH RKVRLVAS

Figure 1

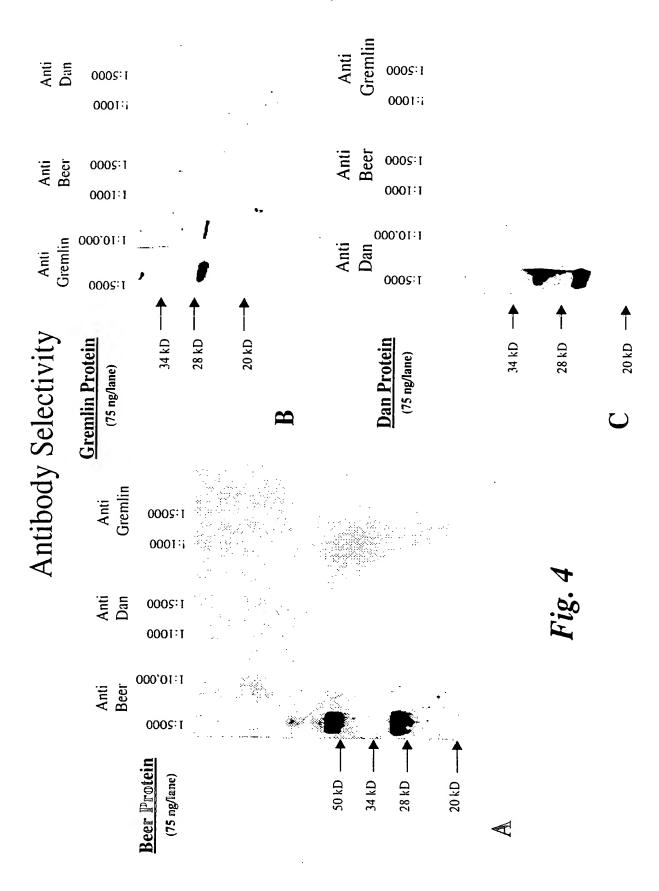
Human Beer Gene Expression by RT-PCR

BBMC genomic s. cerevisiae woukey bone ment here here that was high tich has here had me have here cartilage bone marrow poue osteosarcoma osteoblast pi tui tary thyroid ak. muscle bl acenta thymus uəəjds liver prain beta-actin BEER

Fig. 2

RNA In Situ Hybridization of Mouse Embryo Sections

BNSDOCID: <WO___0032773A1_I_>



Evaluation of Beer binding to BMP family members Anti-FLAG Immunoprecipitation

Beer + + siMR.²
BMP-5 + - control

*Anti-BMP-5 western blot

*Anti-BMP-6 western blot

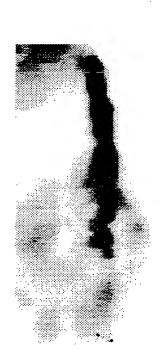
BMP-4 C - + C

Beer - - -
Cerberus - + + -

*Anti-BMP-4 western blot

Fig. 5

BMP-5/Beer Dissociation Constant Characterization .75 1.5 7.5 15 30 60 120 nM BMP-5



*Anti-FLAG immunoprecipitation *Anti-BMP-5 western blot

Ionic Disruption of BMP-5/ Beer Binding

ì	Č	•		5.0
NaCI(mM)	200	150	150	BANT STA
Beer	+	+	1	Wester.
BMP-5	+	+.	+	contro

* Anti FLAG immunoprecipitation *Anti BMP-5 western

Fig. 6

SEQUENCE LISTING

	<110>	Brunkow, M	ary E.				
5	•	Galas, Dav	rid J.		•		
	;	Kovacevich	, Brian				
	I	Mulligan,	John T.				
	:	Paeper, Br	yan W.				
	•	Van Ness,	Jeffrey				
10	1	Winkler, D	avid G.				
				ODS FOR INC	REASING		
15	BONE	MINERALIZ	ATION				
	<130>	240083.508					
	(1307)	210003.300					
	<140> 1	US					
	<141>	1999-11-24					
20							
	<160>	41					
	<170>	FastSEQ fo	or Windows V	ersion 3.0			
25	<210>	1					
	<211>	2301					
	<212> 1						
	<213> 1	Homo sapie	n				
20	100	_					
30	<400>						
	agageetgtg c						60
	tggccctgtg to						120
	ggtggcaggc g						180
35	agectecace g						240
	traccodeta co						300
	tcacccgcta c	gryaccyat	aggeegegee	gcagcgccaa	gccggtcacc	gagctggtgt	360

	gctccggcca	gtgcggcccg	gcgcgcctgc	tgcccaacgc	catcggccgc	ggcaagtggt	420
	ggcgacctag	tgggcccgac	ttccgctgca	tccccgaccg	ctaccgcgcg	cagcgcgtgc	480
	agctgctgtg	tcccggtggt	gaggcgccgc	gcgcgcgcaa	ggtgcgcctg	gtggcctcgt	540
	gcaagtgcaa	gcgcctcacc	cgcttccaca	accagtcgga	gctcaaggac	ttcgggaccg	600
5	aggccgctcg	gccgcagaag	ggccggaagc	cgcggccccg	cgcccggagc	gccaaagcca	660
	accaggccga	gctggagaac	gcctactaga	gcccgcccgc	gcccctcccc	accggcgggc	720
	gccccggccc	tgaacccgcg	ccccacattt	ctgtcctctg	cgcgtggttt	gattgtttat	780
	atttcattgt	aaatgcctgc	aacccagggc	agggggctga	gaccttccag	gccctgagga	840
	atcccgggcg	ccggcaaggc	cccctcagc	ccgccagctg	aggggtccca	cggggcaggg	900
10	gagggaattg	agagtcacag	acactgagcc	acgcagcccc	gcctctgggg	ccgcctacct	960
	ttgctggtcc	cacttcagag	gaggcagaaa	tggaagcatt	ttcaccgccc	tggggtttta	1020
	agggagcggt	gtgggagtgg	gaaagtccag	ggactggtta	agaaagttgg	ataagattcc	1080
	cccttgcacc	tcgctgccca	tcagaaagcc	tgaggcgtgc	ccagagcaca	agactggggg	1140
	caactgtaga	tgtggtttct	agtcctggct	ctgccactaa	cttgctgtgt	aaccttgaac	1200
15	tacacaattc	tccttcggga	cctcaatttc	cactttgtaa	aatgagggtg	gaggtgggaa	1260
	taggatctcg	aggagactat	tggcatatga	ttccaaggac	tccagtgcct	tttgaatggg	1320
	cagaggtgag	agagagagag	agaaagagag	agaatgaatg	cagttgcatt	gattcagtgc	1380
	caaggtcact	tccagaattc	agagttgtga	tgctctcttc	tgacagccaa	agatgaaaaa	1440
	caaacagaaa	aaaaaagta	aagagtctat	ttatggctga	catatttacg	gctgacaaac	1500
20	tcctggaaga	agctatgctg	cttcccagcc	tggcttcccc	ggatgtttgg	ctacctccac	1560
	ccctccatct	caaagaaata	acatcatcca	ttggggtaga	aaaggagagg	gtccgagggt	1620
	ggtgggaggg	atagaaatca	catccgcccc	aacttcccaa	agagcagcat	ccctcccccg	1680
	acccatagcc	atgttttaaa	gtcaccttcc	gaagagaagt	gaaaggttca	aggacactgg	1740
	ccttgcaggc	ccgagggagc	agccatcaca	aactcacaga	ccagcacatc	ccttttgaga	1800
25	caccgccttc	tgcccaccac	tcacggacac	atttctgcct	agaaaacagc	ttcttactgc	1860
	tcttacatgt	gatggcatat	cttacactaa	aagaatatta	ttgggggaaa	aactacaagt	1920
	gctgtacata	tgctgagaaa	ctgcagagca	taatagctgc	cacccaaaaa	tctttttgaa	1980
	aatcatttcc	agacaacctc	ttactttctg	tgtagttttt	aattgttaaa	aaaaaaaagt	2040
	tttaaacaga	agcacatgac	atatgaaagc	ctgcaggact	ggtcgttttt	ttggcaattc	2100
30	ttccacgtgg	gacttgtcca	caagaatgaa	agtagtggtt	tttaaagagt	taagttacat	2160
	atttatttc	tcacttaagt	tatttatgca	aaagtttttc	ttgtagagaa	tgacaatgtt	2220
	aatattgctt	tatgaattaa	cagtctgttc	ttccagagtc	cagagacatt	gttaataaag	2280
	acaatgaatc	atgaccgaaa	g				2301

35 <210> 2

<400> 2

5	Met	Gln	Leu	Pro	Leu	Ala	Leu	Cys	Leu	Val	Cys	Leu	Leu	Val	His	Thr
	1				5					10					15	
	Ala	Phe	Arg	Val	Val	Glu	Gly	Gln	Gly	Trp	Gln	Ala	Phe	Lys	Asn	Asp
				20					25					30		
	Ala	Thr	Glu	Ile	Ile	Pro	Glu	Leu	Gly	Glu	Tyr	Pro	Glu	Pro	Pro	Pro
10			35					40					45			
	Glu	Leu	Glu	Asn	Asn	Lys	Thr	Met	Asn	Arg	Ala	Glu	Asn	Gly	Gly	Arq
		50					55					60		_	_	_
	Pro	Pro	His	His	Pro	Phe	Glu	Thr	Lys	Asp	Val	Ser	Glu	Tyr	Ser	Cys
	65					70					75			_	•	80
15	Arg	Glu	Leu	His	Phe	Thr	Arg	Tyr	Val	Thr	Asp	Gly	Pro	Cys	Arg	Ser
					85					90					95	
	Ala	Lys	Pro	Val	Thr	Glu	Leu	Val	Cys	Ser	Gly	Gln	Cys	Gly	Pro	Ala
				100					105					110		
	Arg	Leu	Leu	Pro	Asn	Ala	Ile	Gly	Arg	Gly	Lys	Trp	Trp	Arg	Pro	Ser
20			115					120					125			
	Gly	Pro	Asp	Phe	Arg	Cys	Ile	Pro	Asp	Arg	Tyr	Arg	Ala	Gln	Arg	Val
		130					135					140				
	Gln	Leu	Leu	Cys	Pro	Gly	Gly	Glu	Ala	Pro	Arg	Ala	Arg	Lys	Val	Arg
	145					150					155					160
25	Leu	Val	Ala	Ser	Cys	Lys	Cys	Lys	Arg	Leu	Thr	Arg	Phe	His	Asn	Gln
					165					170					175	
	Ser	Glu	Leu	Lys	Asp	Phe	Gly	Thr	Glu	Ala	Ala	Arg	Pro	Gln	Lys	Gly
				180					185					190	-	-
	Arg	Lys	Pro	Arg	Pro	Arg	Ala	Arg	Ser	Ala	Lys	Ala	Asn	Gln	Ala	Glu
30			195					200					205			
	Leu	Glu	Asn	Ala	Tyr											
		210														

<210> 3
35 <211> 2301
<212> DNA

<213> Homo sapien

<400> 3

	agagcctgtg	ctactggaag	gtggcgtgcc	ctcctctggc	tggtaccatg	cagctcccac	60
5	tggccctgtg	tctcgtctgc	ctgctggtac	acacageett	ccgtgtagtg	gagggctagg	120
	ggtggcaggc	gttcaagaat	gatgccacgg	aaatcatccc	cgagctcgga	gagtaccccg	180
	agcctccacc	ggagctggag	aacaacaaga	ccatgaaccg	ggcggagaac	ggagggcggc	240
	ctccccacca	cccctttgag	accaaagacg	tgtccgagta	cagctgccgc	gagctgcact	300
	tcacccgcta	cgtgaccgat	gggccgtgcc	gcagcgccaa	gccggtcacc	gagctggtgt	360
10	gctccggcca	gtgcggcccg	gcgcgcctgc	tgcccaacgc	catcggccgc	ggcaagtggt	420
	ggcgacctag	tgggcccgac	ttccgctgca	tccccgaccg	ctaccgcgcg	cagcgcgtgc	480
	agctgctgtg	tcccggtggt	gaggcgccgc	gcgcgcgcaa	ggtgcgcctg	gtggcctcgt	540
	gcaagtgcaa	gcgcctcacc	cgcttccaca	accagtcgga	gctcaaggac	ttcgggaccg	600
	aggccgctcg	gccgcagaag	ggccggaagc	cgcggccccg	cgcccggagc	gccaaagcca	660
15	accaggccga	gctggagaac	gcctactaga	gcccgcccgc	gcccctcccc	accggcgggc	720
	gccccggccc	tgaacccgcg	ccccacattt	ctgtcctctg	cgcgtggttt	gattgtttat	780
	atttcattgt	aaatgcctgc	aacccagggc	agggggctga	gaccttccag	gccctgagga	840
	atcccgggcg	ccggcaaggc	cccctcagc	ccgccagctg	aggggtccca	cggggcaggg	900
	gagggaattg	agagtcacag	acactgagcc	acgcagcccc	gcctctgggg	ccgcctacct	960
20	ttgctggtcc	cacttcagag	gaggcagaaa	tggaagcatt	ttcaccgccc	tggggtttta	1020
	agggagcggt	gtgggagtgg	gaaagtccag	ggactggtta	agaaagttgg	ataagattcc	1080
	cccttgcacc	tcgctgccca	tcagaaagcc	tgaggcgtgc	ccagagcaca	agactggggg	1140
	caactgtaga	tgtggtttct	agtcctggct	ctgccactaa	cttgctgtgt	aaccttgaac	1200
	tacacaattc	tccttcggga	cctcaatttc	cactttgtaa	aatgagggtg	gaggtgggaa	1260
25	taggatctcg	aggagactat	tggcatatga	ttccaaggac	tccagtgcct	tttgaatggg	1320
	cagaggtgag	agagagagag	agaaagagag	agaatgaatg	cagttgcatt	gattcagtgc	1380
	caaggtcact	tccagaattc	agagttgtga	tgctctcttc	tgacagccaa	agatgaaaaa	1440
	caaacagaaa	aaaaaagta	aagagtctat	ttatggctga	catatttacg	gctgacaaac	1500
	tcctggaaga	agctatgctg	cttcccagcc	tggcttcccc	ggatgtttgg	ctacctccac	1560
30	ccctccatct	caaagaaata	acatcatcca	ttggggtaga	aaaggagagg	gtccgagggt	1620
	ggtgggaggg	atagaaatca	catccgcccc	aacttcccaa	agagcagcat	ccctcccccg	1680
	acccatagcc	atgttttaaa	gtcaccttcc	gaagagaagt	gaaaggttca	aggacactgg	1740
	ccttgcaggc	ccgagggagc	agccatcaca	aactcacaga	ccagcacatc	ccttttgaga	1800
	caccgccttc	tgcccaccac	tcacggacac	atttctgcct	agaaaacagc	ttcttactgc	1860
35	tcttacatgt	gatggcatat	cttacactaa	aagaatatta	ttgggggaaa	aactacaagt	1920
	gctgtacata	tgctgagaaa	ctgcagagca	taatagctgc	cacccaaaaa	tctttttgaa	1980

	aatcatttcc	agacaacctc	ttactttctg	tgtagttttt	aattgttaaa	aaaaaaagt	2040
	tttaaacaga	agcacatgac	atatgaaagc	ctgcaggact	ggtcgttttt	ttggcaattc	2100
	ttccacgtgg	gacttgtcca	caagaatgaa	agtagtggtt	tttaaagagt	taagttacat	2160
	atttatttc	tcacttaagt	tatttatgca	aaagttttc	ttgtagagaa	tgacaatgtt	2220
5	aatattgctt	tatgaattaa	cagtctgttc	ttccagagtc	cagagacatt	gttaataaag	2280
	acaatgaatc	atgaccgaaa	g				2301
	<210	> 4					
	<211:	> 23					
10	<212:	> PRT					
	<213:	> Homo sapi	en				
	<400						
		Pro Leu A	la Leu Cys I	Leu Val Cys	Leu Leu Val	His Thr	
15	1	5		10		15	
	Ala Phe Arg	y Val Val G	lu Gly				
		20					
	<210:	. 5					
20		> 2301					
20		> 2301 > DNA					
		> Homo sapie	en e				
		Bupi	•••				
	<400:	> 5					
25	agagcctgtg	ctactggaag	gtggcgtgcc	ctcctctggc	tggtaccatg	cagctcccac	60
	tggccctgtg	tctcatctgc	ctgctggtac	acacagcctt	ccgtgtagtg	gagggccagg	120
	ggtggcaggc	gttcaagaat	gatgccacgg	aaatcatccg	cgagctcgga	gagtaccccg	180
	agcctccacc	ggagctggag	aacaacaaga	ccatgaaccg	ggcggagaac	ggagggcggc	240
	ctccccacca	cccctttgag	accaaagacg	tgtccgagta	cagctgccgc	gagctgcact	300
30	tcacccgcta	cgtgaccgat	gggccgtgcc	gcagcgccaa	gccggtcacc	gagctggtgt	360
	gctccggcca	gtgcggcccg	gcgcgcctgc	tgcccaacgc	catcggccgc	ggcaagtggt	420
	ggcgacctag	tgggcccgac	ttccgctgca	tccccgaccg	ctaccgcgcg	cagcgcgtgc	480
	agctgctgtg	tcccggtggt	gaggcgccgc	gcgcgcgcaa	ggtgcgcctg	gtggcctcgt	540
	gcaagtgcaa	gcgcctcacc	cgcttccaca	accagtcgga	gctcaaggac	ttcgggaccg	600
35	aggccgctcg	gccgcagaag	ggccggaagc	cgcggccccg	cgcccggagc	gccaaagcca	660

accaggeega getggagaac geetaetaga geeegeege geeeeteece accggeggge

	gccccggccc	tgaacccgcg	ccccacattt	ctgtcctctg	cgcgtggttt	gattgtttat	780
	atttcattgt	aaatgcctgc	aacccagggc	agggggctga	gaccttccag	gccctgagga	840
	atcccgggcg	ccggcaaggc	cccctcagc	ccgccagctg	aggggtccca	cggggcaggg	900
	gagggaattg	agagtcacag	acactgagcc	acgcagcccc	gcctctgggg	ccgcctacct	960
5	ttgctggtcc	cacttcagag	gaggcagaaa	tggaagcatt	ttcaccgccc	tggggtttta	1020
	agggagcggt	gtgggagtgg	gaaagtccag	ggactggtta	agaaagttgg	ataagattcc	1080
	cccttgcacc	tcgctgccca	tcagaaagcc	tgaggcgtgc	ccagagcaca	agactggggg	1140
	caactgtaga	tgtggtttct	agtcctggct	ctgccactaa	cttgctgtgt	aaccttgaac	1200
	tacacaattc	tccttcggga	cctcaatttc	cactttgtaa	aatgagggtg	gaggtgggaa	1260
10 .	taggatctcg	aggagactat	tggcatatga	ttccaaggac	tccagtgcct	tttgaatggg	1320
	cagaggtgag	agagagagag	agaaagagag	agaatgaatg	cagttgcatt	gattcagtgc	1380
				tgctctcttc			1440
				ttatggctga			1500
				tggcttcccc			1560
15				ttggggtaga			1620
				aacttcccaa			1680
		•		gaagagaagt			1740
				aactcacaga			1800
				atttctgcct			1860
20				aagaatatta			1920
				taatagctgc			1980
				tgtagttttt			2040
				ctgcaggact			2100
				agtagtggtt			2160
25				aaagtttttc			2220
	aatattgctt	tatgaattaa	cagtctgttc	ttccagagtc	cagagacatt	gttaataaag	2280
	acaatgaatc	atgaccgaaa	g				2301

<210> 6

30 <211> 213

<212> PRT

<213> Homo sapien

<400> 6

Met Gln Leu Pro Leu Ala Leu Cys Leu Ile Cys Leu Leu Val His Thr

1 5 10 15

	Ala	a Phe	e Arg	Va]	l Vai	l Glu	Gly	/ Glr	Gly 25	/ Trp	Glr	ı Ala	a Phe		s Ası	n Asp	
	Ala	Thi	: Glu		: Ile	e Aro	Glu	ı I.a.ı		. Cl.	. m	_		30		o Pro	
			35			9	OIO	40	Gly	GIU	ı ıyr	Pro		Pro) Pro	Pro	
5	Glu	Leu	Glu	Asn	Asr	ı Lys	Thr		Asn	Ara	בומי	C1.	45	a 3		/ Arg	
		50				-	55			• ••• 9	ALG	60	ı ASN	GIY	, Gly	/ Arg	
	Pro	Pro	His	His	Pro	Phe	Glu	Thr	Lys	Asp	Val		· Glu	Tur			
	65					70			-	•	75		GIU	TYL	361	80	
	Arg	Glu	Leu	His	Phe	Thr	Arg	Tyr	Val	Thr		Gly	Pro	Cvs	Ara	Ser	
10					85					90					95		
	Ala	Lys	Pro	Val	Thr	Glu	Leu	Val	Cys	Ser	Gly	Gln	Cys	Gly	Pro	Ala	
				100					105					110			
	Arg	Leu	Leu	Pro	Asn	Ala	Ile	Gly	Arg	Gly	Lys	Trp	Trp	Arg	Pro	Ser	
15	G1	_	115					120					125				
15	GIY	Pro	Asp	Phe	Arg	Cys		Pro	Asp	Arg	Tyr	Arg	Ala	Gln	Arg	Val	
	Gln	130	T 011	C	D	~ 1	135					140					
	145	Dea	neu	cys	PIO	Gly	Gly	Glu	Ala	Pro		Ala	Arg	Lys	Val	Arg	
		Val	Ala	Ser	Cvs	150	Cvc	T	>	•	155					160	
20				001	165	Lys	Cys	гуs	Arg		Thr	Arg	Phe	His	Asn	Gln	
	Ser	Glu	Leu	Lys		Phe	Glv	Thr	Glu	170	77 -		_		175		
				180	•		1		185	A10	Ala	Arg			Lys	Gly	
	Arg	Lys	Pro .	Arg	Pro	Arg .	Ala			Ala	Lvs	Δla	λαη	190	7.7	~ 1	
			195					200			-,5	mia	205	GIII	Ala	Glu	
25	Leu	Glu	Asn I	Ala	Tyr												
	:	210															
			10> 7														
20	,		11> 2														
30			12> [
		<2	13> F	Iomo	sap	ien											
		<4(00> 7	,													
	agago				מפטנ	ם מדה	ימרת+	- 000	at = :								
35	tggcc	ctgt	gto	tcat	ctar	5 CFC	ictar	itac	2000	.ccto	gc t	ggt	accat	g ca	agcto	ccac	60
	tggcc ggtgg	cago	gc gt	tcaa	gaat	aat	acca	cac	acac	agcc	:	cgt	gtagt	g ga	aggg	ccagg	120
	_			-	J \	- 300		33	aaat	Cato	cg c	gago	ctcgg	ia ga	agtad	cccg	180

	agcetecace	ggagctggag	aacaacaaga	ccatgaaccg	ggcggagaac	ggagggcggc	240
	ctcccacca	ccctttgag	accaaagaco	, tgtccgagta	cagetgeege	gagctgcact	300
	tcacccgcta	cgtgaccgat	gggccgtgcc	gcagcgccaa	gccggtcacc	gagctggtgt	360
	gctccggcca	gtgcggcccg	gcgcgcctgc	tgcccaacgc	catcggccgc	ggcaagtggt	420
5	ggcgacctag	tgggcccgac	ttccgctgca	tccccgaccg	ctaccgcgcg	cagcgcgtgc	480
	agctgctgtg	tcccggtggt	gaggcgccgc	gcgcgcgcaa	ggtgcgcctg	gtggcctcgt	540
	gcaagtgcaa	gcgcctcacc	cgcttccaca	accagtcgga	gctcaaggac	ttcgggaccg	600
	aggccgctcg	gccgcagaag	ggccggaagc	cgcggccccg	cgcccggagc	gccaaagcca	660
	accaggccga	gctggagaac	gcctactaga	gcccgcccgc	gcccctcccc	accggcgggc	720
10	gccccggccc	tgaacccgcg	ccccacattt	ctgtcctctg	cgcgtggttt	gattgtttat	780
	atttcattgt	aaatgcctgc	aacccagggc	agggggctga	gaccttccag	gccctgagga	840
	atcccgggcg	ccggcaaggc	cccctcagc	ccgccagctg	aggggtccca	cggggcaggg	900
	gagggaattg	agagtcacag	acactgagcc	acgcagcccc	gcctctgggg	ccgcctacct	960
	ttgctggtcc	cacttcagag	gaggcagaaa	tggaagcatt	ttcaccgccc	tggggtttta	1020
15	agggagcggt	gtgggagtgg	gaaagtccag	ggactggtta	agaaagttgg	ataagattcc	1080
	cccttgcacc	tcgctgccca	tcagaaagcc	tgaggcgtgc	ccagagcaca	agactggggg	1140
	caactgtaga	tgtggtttct	agtcctggct	ctgccactaa	cttgctgtgt	aaccttgaac	1200
	tacacaattc	tccttcggga	cctcaatttc	cactttgtaa	aatgagggtg	gaggtgggaa	1260
	taggateteg	aggagactat	tggcatatga	ttccaaggac	tccagtgcct	tttgaatggg	1320
20	cagaggtgag	agagagagag	agaaagagag	agaatgaatg	cagttgcatt	gattcagtgc	1380
	caaggtcact	tccagaattc	agagttgtga	tgctctcttc	tgacagccaa	agatgaaaaa	1440
	caaacagaaa	aaaaaagta	aagagtctat	ttatggctga	catatttacg	gctgacaaac	1500
	tcctggaaga	agctatgctg	cttcccagcc	tggcttcccc	ggatgtttgg	ctacctccac	1560
	ccctccatct	caaagaaata	acatcatcca	ttggggtaga	aaaggagagg	gtccgagggt	1620
25	ggtgggaggg	atagaaatca	catccgcccc	aacttcccaa	agagcagcat	ccctcccccg	1680
	acccatagcc	atgttttaaa	gtcaccttcc	gaagagaagt	gaaaggttca	aggacactgg	1740
	ccttgcaggc	ccgagggagc	agccatcaca	aactcacaga	ccagcacatc	ccttttgaga	1800
	caccgccttc	tgcccaccac	tcacggacac	atttctgcct	agaaaacagc	ttcttactgc	1860
	tcttacatgt	gatggcatat	cttacactaa	aagaatatta	ttgggggaaa	aactacaagt	1920
30	gctgtacata	tgctgagaaa	ctgcagagca	taatagctgc	cacccaaaaa	tctttttgaa	1980
	aatcatttcc	agacaacctc	ttactttctg	tgtagttttt	aattgttaaa	aaaaaaagt	2040
	tttaaacaga	agcacatgac	atatgaaagc	ctgcaggact	ggtcgttttt	ttggcaattc	2100
	ttccacgtgg	gacttgtcca	caagaatgaa	agtagtggtt	tttaaagagt	taagttacat	2160
	atttatttc	tcacttaagt	tatttatgca	aaagtttttc	ttgtagagaa	tgacaatgtt	2220
35	aatattgctt	tatgaattaa d	cagtctgttc	ttccagagtc	cagagacatt	gttaataaag	2280
	acaatgaatc	atgaccgaaa q	3				2301

			<210	> 8												
		•	<211:	> 21	3											
			<212	> PR	r											
5		•	<213:	> Hor	no sa	pien	1									
		<	400>	8												
	Met	Glr	ı Leı	Pro	Leu	Ala	Leu	ı Cvs	Lei	ı Val	Cve		T	**- *	•••	Thr
	1				5			- 3 -		10	- Cys	neu	neu	vai		Thr
10	Ala	Phe	Arc	. Val	Val	Glu	Glv	G)n	Gla		· C1-		D1	_	15	Asp
			_	20			1	04	25	111	GII	Ala	Pne		Asn	Asp
	Ala	Thr	Glu	Ile	Ile	Ara	Glu	T.e.u			T	D	~ .	30	_	Pro
			35	_		5	O.L.	40	GIY	GIU	ıyı	Pro		Pro	Pro	Pro
	Glu	Leu		Asn	Asn	Lve	Thr		7~~	.			45			
15		50			11511	БуЗ	55	Met	ASN	Arg	Ala		Asn	Gly	Gly	Arg
	Pro		Hie	Hic	Pro	Dha		m ⊱	_			60				
	65		5	*****	FIO	70	GIU	Inr	Lys	Asp		Ser	Glu	Tyr	Ser	Cys
		Glu	Len	uic	Dho			_			75					80
	9	Oiu	шец	піз		Int	Arg	Tyr	Val		Asp	Gly	Pro	Cys	Arg	Ser
20	בומ	Lva	D~0	1/2]	85	a 3	_			90					95	
	AIG	пуѕ	PIO	vai	Inr	Glu	Leu	Val	Cys	Ser	Gly	Gln	Cys	Gly	Pro	Ala
	λ	T	.	100	_				105					110		
	Arg	ьeu		Pro	Asn	Ala	Ile	Gly	Arg	Gly	Lys	Trp	Trp	Arg	Pro	Ser
	0.1	_	115					120					125			
26	GIY	Pro	Asp	Phe	Arg	Cys	Ile	Pro	Asp	Arg	Tyr	Arg	Ala	Gln	Arg	Val
25		130					135					140				
	Gln	Leu	Leu	Cys	Pro	Gly	Gly	Glu	Ala	Pro	Arg	Ala	Arg	Lys	Val	Arg
	145					150					155					160
	Leu	Val	Ala	Ser	Cys	Lys	Cys	Lys	Arg	Leu	Thr	Arg	Phe	His	Asn	Gln
					165					170					175	
30	Ser	Glu	Leu	Lys	Asp	Phe	Gly	Thr	Glu	Ala	Ala	Arg	Pro	Gln	Lys	Gly
				180					185					190	-	•

Arg Lys Pro Arg Pro Arg Ala Arg Ser Ala Lys Ala Asn Gln Ala Glu

205

200

35

195

Leu Glu Asn Ala Tyr

210

```
<210> 9
             <211> 642
             <212> DNA
             <213> Cercopithecus pygerythrus
  5
            <400> 9
      atgcagetee cactggeest gtgtettgte tgeetgetgg tacaegeage etteegtgta
                                                                               60
      gtggagggcc aggggtggca ggccttcaag aatgatgcca cggaaatcat ccccgagctc
                                                                              120
      ggagagtacc ccgagcctcc accggagctg gagaacaaca agaccatgaa ccgggcggag
                                                                              180
      aatggagggc ggcctcccca ccacccttt gagaccaaag acgtgtccga gtacagctgc
 10
                                                                              240
      cgagagetge actteaceeg etaegtgace gatgggeegt geegeagege caageeagte
                                                                              300
      accgagttgg tgtgctccgg ccagtgcggc ccggcacgcc tgctgcccaa cgccatcggc
                                                                              360
      cgcggcaagt ggtggcgccc gagtgggccc gacttccgct gcatccccga ccgctaccgc
                                                                              420
      gcgcagcgtg tgcagctgct gtgtcccggt ggtgccgcgc cgcgcgcgcg caaggtgcgc
                                                                              480
      ctggtggcct cgtgcaagtg caagcgcctc acccgcttcc acaaccagtc ggagctcaag
15
                                                                              540
      gacttcggtc ccgaggccgc tcggccgcag aagggccgga agccgcggcc ccgcgcccgg
                                                                              600
      ggggccaaag ccaatcaggc cgagctggag aacgcctact ag
                                                                              642
            <210> 10
20
            <211> 213
            <212> PRT
            <213> Cercopithecus pygerythrus
            <400> 10
      Met Gln Leu Pro Leu Ala Leu Cys Leu Val Cys Leu Leu Val His Ala
25
       1
                       5
                                           10
                                                               15
      Ala Phe Arg Val Val Glu Gly Gln Gly Trp Gln Ala Phe Lys Asn Asp
                                      25
     Ala Thr Glu Ile Ile Pro Glu Leu Gly Glu Tyr Pro Glu Pro Pro
30
              35
                                  40
                                                       45
     Glu Leu Glu Asn Asn Lys Thr Met Asn Arg Ala Glu Asn Gly Gly Arg
          50
                              55
     Pro Pro His His Pro Phe Glu Thr Lys Asp Val Ser Glu Tyr Ser Cys
     65
                          70
                                              75
     Arg Glu Leu His Phe Thr Arg Tyr Val Thr Asp Gly Pro Cys Arg Ser
35
                      85
                                          90
                                                               95
```

	Ala Lys Pro Val Thr Glu Leu Val Cys Ser Gly Gln Cys Gly Pro Ala	
	100 105 110	
	Arg Leu Leu Pro Asn Ala Ile Gly Arg Gly Lys Trp Trp Arg Pro Ser	
_	115 120 125	
5	Gly Pro Asp Phe Arg Cys Ile Pro Asp Arg Tyr Arg Ala Gln Arg Val	
	130 135 140	
	Gln Leu Leu Cys Pro Gly Gly Ala Ala Pro Arg Ala Arg Lys Val Arg	
	145 150 155	
10	Leu Val Ala Ser Cys Lys Cys Lys Arg Leu Thr Arg Phe His Asn Gln	
10	165 170	
	Ser Glu Leu Lys Asp Phe Gly Pro Glu Ala Ala Arg Pro Gln Lys Gly	
	180 185 190	
	Arg Lys Pro Arg Pro Arg Ala Arg Gly Ala Lys Ala Asn Gln Ala Glu	
15	200 205	
13	Leu Glu Asn Ala Tyr	
	210	
	-210	
	<210> 11	
20	<211> 638 <212> DNA	
	<213> Mus musculus	
	<400> 11	
25	atgragger agggtggs aggettatt tgeetacttg tgeacgetge ettetgtget	60
	gtggagggcc aggggtggca agcettcagg aatgatgcca cagaggtcat eccagggett	120
	ggagagtacc ccgagcetec tectgagaac aaccagacca tgaaccggge ggagaatgga	180
	ggcagacete eccaceatee etatgacgee aaaggtgtgt eegagtacag etgeegegag	240
	ctgcactaca cccgcttcct gacagacggc ccatgccgca gcgccaagcc ggtcaccgag	300
30	ttggtgtgct ccggccagtg cggccccgcg cggctgctgc ccaacgccat cgggcgcgtg	360
	aagtggtggc gcccgaacgg accggatttc cgctgcatcc cggatcgcta ccgcgcgcag	420
	cgggtgcagc tgctgtgccc cgggggcgcg gcgccgcgct cgcgcaaggt gcgtctggtg	480
	gcctcgtgca agtgcaagcg cctcacccgc ttccacaacc agtcggagct caaggacttc	540
	gggccggaga ccgcggggc gcagaagggt cgcaagccgc ggcccggcgc ccggggagcc	600
35	aaagccaacc aggcggagct ggagaacgcc tactagag	638

<210> 12

<211> 211 <212> PRT <213> Mus musculus

5 <400> 12 Met Gln Pro Ser Leu Ala Pro Cys Leu Ile Cys Leu Leu Val His Ala 10 Ala Phe Cys Ala Val Glu Gly Gln Gly Trp Gln Ala Phe Arg Asn Asp 25 Ala Thr Glu Val Ile Pro Gly Leu Gly Glu Tyr Pro Glu Pro Pro . 10 35 40 45 Glu Asn Asn Gln Thr Met Asn Arg Ala Glu Asn Gly Gly Arg Pro Pro 55 His His Pro Tyr Asp Ala Lys Asp Val Ser Glu Tyr Ser Cys Arg Glu 15 65 70 75 Leu His Tyr Thr Arg Phe Leu Thr Asp Gly Pro Cys Arg Ser Ala Lys 85 Pro Val Thr Glu Leu Val Cys Ser Gly Gln Cys Gly Pro Ala Arg Leu 105 Leu Pro Asn Ala Ile Gly Arg Val Lys Trp Trp Arg Pro Asn Gly Pro 20 115 120 Asp Phe Arg Cys Ile Pro Asp Arg Tyr Arg Ala Gln Arg Val Gln Leu 135 Leu Cys Pro Gly Gly Ala Ala Pro Arg Ser Arg Lys Val Arg Leu Val 25 145 150 155 Ala Ser Cys Lys Cys Lys Arg Leu Thr Arg Phe His Asn Gln Ser Glu 165 170 Leu Lys Asp Phe Gly Pro Glu Thr Ala Arg Pro Gln Lys Gly Arg Lys 180 185 Pro Arg Pro Gly Ala Arg Gly Ala Lys Ala Asn Gln Ala Glu Leu Glu 30 195 200 205 Asn Ala Tyr 210

35 <210> 13 <211> 674

<212> DNA

<213> Rattus norvegicus

<400> 13

_							
5	gaggaccgag	tgcccttcct	ccttctggca	ccatgcagct	ctcactagec	ccttgccttg	60
	cctgcctgct	tgtacatgca	gccttcgttg	ctgtggagag	ccaggggtgg	caagccttca	120
	agaatgatgc	cacagaaatc	atcccgggac	tcagagagta	cccagagcct	cctcaggaac	180
	tagagaacaa	ccagaccatg	aaccgggccg	agaacggagg	cagacccccc	Caccatcott	240
	atgacaccaa	agacgtgtcc	gagtacagct	gccgcgagct	gcactacacc	CGCttCgtga	300
10	ccgacggccc	gtgccgcagt	gccaagccgg	tcaccgagtt	ggtgtgctcg	ggccagtgcg	360
	gccccgcgcg	gctgctgccc	aacgccatcg	ggcgcgtgaa	gtggtggcqc	CCGaacggac	420
	ccgacttccg	ctgcatcccg	gatcgctacc	gcgcgcagcg	ggtgcagctq	Ctataccca	480
	gcggcgcggc	gccgcgctcg	cgcaaggtgc	gtctggtggc	ctcgtgcaag	tgcaagcgcc	540
	tcacccgctt	ccacaaccag	tcggagctca	aggacttcgg	acctgagacc	acacaaccac	600
15	agaagggtcg	caagccgcgg	ccccgcgccc	ggggagccaa	agccaaccag	acaaaactaa	660
	agaacgccta	ctag	•		5	2-22-2-199	674
							J / 4

<210> 14

<211> 213

20 <212> PRT

<213> Rattus norvegicus

<400> 14

Met Gln Leu Ser Leu Ala Pro Cys Leu Ala Cys Leu Leu Val His Ala 25 1 5 10 15 Ala Phe Val Ala Val Glu Ser Gln Gly Trp Gln Ala Phe Lys Asn Asp Ala Thr Glu Ile Ile Pro Gly Leu Arg Glu Tyr Pro Glu Pro Pro Gln 35 40 30 Glu Leu Glu Asn Asn Gln Thr Met Asn Arg Ala Glu Asn Gly Gly Arg 50 60 Pro Pro His His Pro Tyr Asp Thr Lys Asp Val Ser Glu Tyr Ser Cys 75 Arg Glu Leu His Tyr Thr Arg Phe Val Thr Asp Gly Pro Cys Arg Ser 35 85 90 Ala Lys Pro Val Thr Glu Leu Val Cys Ser Gly Gln Cys Gly Pro Ala

				100					105					110			
	Arg	Leu	Leu	Pro	Asn	Ala	Ile	Gly	Arg	Val	Lys	Trp	Trp	Ara	Pro	Asn	
			115					120			-	•	125	9	110	ASII	
	Gly	Pro	Asp	Phe	Arg	Cys	Ile	Pro	Asp	Arg	Tyr	Arg	Ala	Gln	Δτα	Wa l	
5		130					135			_	-	140		01	n. g	Vai	
	Gln	Leu	Leu	Cys	Pro	Gly	Gly	Ala	Ala	Pro	Ara	Ser	Ara	Lve	Val	A ~~ ~	
	145					150					155		5	2,5	VUI		
	Leu	Val	Ala	Ser	Cys	Lys	Cys	Lys	Arg	Leu		Arg	Phe	Hic	λαπ	160	
					165				•	170		3	- 1.0	3	175	GIII	
10	Ser	Glu	Leu	Lys	Asp	Phe	Gly	Pro	Glu	Thr	Ala	Arg	Pro	Gln	175	C1	
				180					185			9	-10	190	БУБ	GIY	
	Arg	Lys	Pro	Arg	Pro	Arg	Ala	Arg	Gly	Ala	Lvs	Al,a	Acn	Gla	אן ה	C1	
			195					200			•		205	O 111	AIG	GIU	
	Leu	Glu	Asn	Ala	Tyr								205				
15		210															
		<2	10>	15													
		<2	11>	532													
		<2	12>	DNA													
20		<2	13> :	Bos 1	toru	s											
		<40	00>	15													
	agaat	gat	gc ca	acaga	aaato	ato	cccg	gagc	tggg	gogag	gta d	cccc	agco	et ei	. acc:	20200	
	tgaac	caaca	aa ga	accat	gaad	cgg	ggcgg	gaga	acgo	jaggo	gag a	accto	CCC	ac c	-gccc	zgagc	60
25	agaco	aaag	ga co	gcct	cgaç	tac	agct	gcc	ggga	gcto	aca d	itte	accc	ic ta	acct	2222	120
	atggg	ccgt	g co	gcag	geged	aag	gccgc	jtca	ccga	gcto	agt c	atact	Caac	יי כי	acec	raccy	180
	cggcg	cgcc	t go	tgcc	caac	gco	catco	gcc	gcgg	caac	ita c	ataac	acco	a ac	recec	ggcc	
	actto	cgct	g ca	tccc	cgac	cgc	tacc	gcg	cgca	gege	iat c	icago	rarr	a to	rt cot	acceg	300
	gcgcg	gcgc	c go	gcgc	gcgc	aag	gtgc	gcc	tggt	ggcc	itc o	itaca	agto	.g .c	,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,	ggcg	
30	ctcgc	ttcc	a ca	acca	gtco	gag	rctca	.agg	actt	caaa	י ב	aaaa	ccac	,c ac	acgc	ccca	420
	cgggc	cgga	a go	tgcg	gccc	cgc	gccc	ggg	gcac	caaa	ומכ כ	acc.	aaaa	e es	gccg	Caaa	480
								_			J = C	-500	3336	.c ya	•		532
		<21	0 > 1	6													
		<21	1> 1	76													
35		<21	2> P	RT													
		<21	3 > B	os t	orus												

```
<400> 16
        Asn Asp Ala Thr Glu Ile Ile Pro Glu Leu Gly Glu Tyr Pro Glu Pro
                         5
       Leu Pro Glu Leu Asn Asn Lys Thr Met Asn Arg Ala Glu Asn Gly Gly
                                        25
                                                             30
       Arg Pro Pro His His Pro Phe Glu Thr Lys Asp Ala Ser Glu Tyr Ser
                                    40
       Cys Arg Glu Leu His Phe Thr Arg Tyr Val Thr Asp Gly Pro Cys Arg
 10
           50
                                55
                                                    60
       Ser Ala Lys Pro Val Thr Glu Leu Val Cys Ser Gly Gln Cys Gly Pro
                                                75
                                                                    8.0
       Ala Arg Leu Leu Pro Asn Ala Ile Gly Arg Gly Lys Trp Trp Arg Pro
                       85
                                            90
       Ser Gly Pro Asp Phe Arg Cys Ile Pro Asp Arg Tyr Arg Ala Gln Arg
 15
                                       105
                                                            110
      Val Gln Leu Cys Pro Gly Gly Ala Ala Pro Arg Ala Arg Lys Val
               115
                                   120
      Arg Leu Val Ala Ser Cys Lys Cys Lys Arg Leu Thr Arg Phe His Asn
 20
          130
                               135
                                                    140
      Gln Ser Glu Leu Lys Asp Phe Gly Pro Glu Ala Ala Arg Pro Gln Thr
                           150
                                               155
      Gly Arg Lys Leu Arg Pro Arg Ala Arg Gly Thr Lys Ala Ser Arg Ala
                       165
                                           170
25
            <210> 17
            <211> 35828
            <212> DNA
            <213> Mus musculus
30
            <220>
            <221> misc_feature
            <222> (1)...(35828)
            <223> n = A,T,C or G
35
```

<400> 17

	cgcgttttgg	tgagcagcaa	tattgcgctt	cgatgagcct	tggcgttgag	attgatacct	60
	ctgctgcaca	aaaggcaatc	gaccgagctg	gaccagcgca	ttcgtgacac	cgtctccttc	120
	gaacttattc	gcaatggagt	gtcattcatc	aaggacngcc	tgatcgcaaa	tggtgctatc	180
	cacgcagcgg	caatcgaaaa	ccctcagccg	gtgaccaata	tctacaacat	cagccttggt	240
5	atcctgcgtg	atgagccagc	gcagaacaag	gtaaccgtca	gtgccgataa	gttcaaagtt	300
	aaacctggtg	ttgataccaa	cattgaaacg	ttgatcgaaa	acgcgctgaa	aaacgctgct	360
	gaatgtgcgg	cgctggatgt	cacaaagcaa	atggcagcag	acaagaaagc	gatggatgaa	420
	ctggcttcct	atgtccgcac	ggccatcatg	atggaatgtt	tccccggtgg	tgttatctgg	480
	cagcagtgcc	gtcgatagta	tgcaattgat	aattattatc	atttgcgggt	cctttccggc	540
10	gatccgcctt	gttacggggc	ggcgacctcg	cgggttttcg	ctatttatga	aaattttccg	600
	gtttaaggcg	tttccgttct	tcttcgtcat	aacttaatgt	ttttatttaa	aataccctct	660
	gaaaagaaag	gaaacgacag	gtgctgaaag	cgagcttttt	ggcctctgtc	gtttcctttc	720
	tctgtttttg	tccgtggaat	gaacaatgga	agtcaacaaa	aagcagagct	tatcgatgat	780
	aagcggtcaa	acatgagaat	tcgcggccgc	ataatacgac	tcactatagg	gatcgacgcc	840
15	tactccccgc	gcatgaagcg	gaggagctgg	actccgcatg	cccagagacg	cccccaacc	900
	cccaaagtgc	ctgacctcag	cctctaccag	ctctggcttg	ggcttgggcg	gggtcaaggc	960
	taccacgttc	tcttaacagg	tggctgggct	gtctcttggc	cgcgcgtcat	gtgacagctg	1020
	cctagttctg	cagtgaggtc	accgtggaat	gtctgccttc	gttgccatgg	caacgggatg	1080
	acgttacaat	ctgggtgtgg	agcttttcct	gtccgtgtca	ggaaatccaa	ataccctaaa	1140
20	ataccctaga	agaggaagta	gctgagccaa	ggctttcctg	gcttctccag	ataaagtttg	1200
	acttagatgg	aaaaaaacaa	aatgataaag	acccgagcca	tctgaaaatt	cctcctaatt	1260
	gcaccactag	gaaatgtgta	tattattgag	ctcgtatgtg	ttcttattt	aaaaagaaaa	1320
	ctttagtcat	gttattaata	agaatttctc	agcagtggga	gagaaccaat	attaacacca	1380
	agataaaagt	tggcatgatc	cacattgcag	gaagatccac	gttgggtttt	catgaatgtg	1440
25	aagaccccat	ttattaaagt	cctaagctct	gtttttgcac	actaggaagc	gatggccggg	1500
	atggctgagg	ggctgtaagg	atctttcaat	gtcttacatg	tgtgtttcct	gtcctgcacc	1560
	taggacctgc	tgcctagcct	gcagcagagc	cagaggggtt	tcacatgatt	agtctcagac	1620
	acttgggggc	aggttgcatg	tactgcatcg	cttatttcca	tacggagcac	ctactatgtg	1680
	tcaaacacca	tatggtgttc	actcttcaga	acggtggtgg	tcatcatggt	gcatttgctg	1740
30	acggttggat	tggtggtaga	gagctgagat	atatggacgc	actcttcagc	attctgtcaa	1800
	cgtggctgtg	cattcttgct	cctgagcaag	tggctaaaca	gactcacagg	gtcagcctcc	1860
	agctcagtcg	ctgcatagtc	ttagggaacc	tctcccagtc	ctccctacct	caactatcca	1920
	agaagccagg	gggcttggcg	gtctcaggag	cctgcttgct	gggggacagg	ttgttgagtt	1980
	ttatctgcag	taggttgcct	aggcatagtg	tcaggactga	tggctgcctt	ggagaacaca	2040
35	tectttgece	tctatgcaaa	tctgaccttg	acatgggggc	gctgctcagc	tgggaggatc	2100
	aactgcatac	ctaaagccaa	gcctaaagct	tcttcgtcca	cctgaaactc	ctggaccaag	2160

	gggcttccgg	cacatcctct	caggccagtg	agggagtctg	tgtgagctgc	actttccaat	2220
	ctcagggcgt	gagaggcaga	gggaggtggg	ggcagagcct	tgcagctctt	tcctcccatc	2280
	tggacagcgc	tctggctcag	cagcccatat	gagcacaggc	acatccccac	cccaccccca	2340
	cctttcctgt	cctgcagaat	ttaggctctg	ttcacggggg	999999999	ggggcagtcc	2400
5	tatcctctct	taggtagaca	ggactctgca	ggagacactg	ctttgtaaga	tactgcagtt	2460
	taaatttgga	tgttgtgagg	ggaaagcgaa	gggcctcttt	gaccattcag	tcaaggtacc	2520
	ttctaactcc	catcgtattg	gggggctact	ctagtgctag	acattgcaga	gagcctcaga	2580
	actgtagtta	ccagtgtggt	aggattgatc	cttcagggag	cctgacatgt	gacagttcca	2640
	ttcttcaccc	agtcaccgaa	catttattca	gtacctaccc	cgtaacaggc	accgtagcag	2700
10	gtactgaggg	acggaccact	caaagaactg	acagaccgaa	gccttggaat	ataaacacca	2760
	aagcatcagg	ctctgccaac	agaacactct	ttaacactca	ggccctttaa	cactcaggac	2820
	ccccaccccc	accccaagca	gttggcactg	ctatccacat	tttacagaga	ggaaaaacta	2880
	ggcacaggac	gatataagtg	gcttgcttaa	gcttgtctgc	atggtaaatg	gcagggctgg	2940
	attgagaccc	agacattcca	actctagggt	ctattttct	ttttctcgt	tgttcgaatc	3000
15			caggctagcc				3060
			tgtgctacca				3120
			tgatagcagc				3180
			acaggcgaat				3240
			gaccacaagt				3300
20			gtcatgagat				3360
			gaggctcgga				3420
			cctcccatcc				3480
			cgtcctgaat				3540
			cgaggattct				3600
25			cagtgcttac				3660
			taaggaacgt				3720
			agaggccccc				3780
			taagtgtcct				3840
			caatggataa				3900
30			taattgagtg				3960
			ataggttgat				4020
			gggtctcttc				4080
			ggagttctgc				4140
			gcttttatgt				4200
35			ctgccacatc				4260
	ccagaattcc	cccagtgggg	ctttcctacc	cttttattgg	ctaggcattc	atgagtggtc	4320

	acctcgccag	aggaatgagt	ggccacgact	ggctcagggt	cagcagccta	gagatactgg	4380
						ctgaatgaga	4440
	gctggctagt	ggtcagacag	gacagaaggc	tgagagggtc	acagggcaga	tgtcagcaga	4500
			tgggggaggg				4560
5			ctgggtacac				4620
			cttacagaag				4680
	tttggctctt	ggtggacggt	gcatactgct	gtatcagctc	aagagctcat	tcacgaatga	4740
	acacacacac	acacacacac	acacacacac	acacaagcta	attttgatat	gccttaacta	4800
	gctcagtgac	tgggcatttc	tgaacatccc	tgaagttagc	acacatttcc	ctctggtgtt	4860
10	cctggcttaa	caccttctaa	atctatattt	tatctttgct	gccctgttac	cttctgagaa	4920
	gcccctaggg	ccacttccct	tcgcacctac	attgctggat	ggtttctctc	ctgcagctct	4980
	taaatctgat	ccctctgcct	ctgagccatg	ggaacagccc	aataactgag	ttagacataa	5040
	aaacgtctct	agccaaaact	tcagctaaat	ttagacaata	aatcttactg	gttgtggaat	5100
	ccttaagatt	cttcatgacc	tccttcacat	ggcacgagta	tgaagcttta	ttacaattgt	5160
15			taaaaagcca				5220
			tgtgcacctt				5280
			tcacatgtta				5340
			gctgttccag				5400
			aggaggtagg				5460
20			gctgtacatt				5520
			cctcttaatc				5580
			actctggata				5640
			acatggcagt				5700
			tctttttgga				5760
25			gatctttggt				5820
			tatcttacca				5880
			ggaccatgtc				5940
			gctgaacaaa				6000
• •			taaaatggat				6060
30			tgctaagata				6120
			aatccattac				6180
			ttggcgacta				6240
			gggcagagtg				6300
			gcaaactggc				6360
35			ttaacatata				6420
	tagatgccaa	ttttaagccc	ccacatgcac	atggacaagt	gtgcgtttga	acacacatat	6480

	gcactcatgt	gaaccaggca	tgcacactcg	ggcttatcac	acacataatt	tgaaagagag	6540
	agtgagagag	gagagtgcac	attagagttc	acaggaaagt	gtgagtgagc	acacccatgc	6500
	acacagacat	gtgtgccagg	gagtaggaaa	ggagcctggg	tttgtgtata	agagggagcc	6660
	atcatgtgtt	tctaaggagg	gcgtgtgaag	gaggcgttgt	gtgggctggg	actggagcat	6720
5	ggttgtaact	gagcatgctc	cctgtgggaa	acaggagggt	ggccaccctg	cagagggtcc	6780
	cactgtccag	cgggatcagt	aaaagcccct	gctgagaact	ttaggtaata	gccagagaga	6840
	gaaaggtagg	aaagtggggg	gactcccatc	tctgatgtag	gaggatctgg	gcaagtagag	6900
	gtgcgtttga	ggtagaaaga	ggggtgcaga	ggagatgctc	ttaattctgg	gtcagcagtt	6960
	tctttccaaa	taatgcctgt	gaggaggtgt	aggtggtggc	cattcactca	ctcagcagag	7020
10	ggatgatgat	gcccggtgga	tgctggaaat	ggccgagcat	caaccctggc	tctggaagaa	7080
	ctccatcttt	cagaaggaga	gtggatctgt	gtatggccag	cggggtcaca	ggtgcttggg	7140
		gactcctagc					7200
		ctttgtttct					7260
		tcaatcttgg					7320
15		tgtaagcaag					7380
		tgagttccgg					7440
	agctagcggc	aagggtagag	ggcgagctcc	ctgtgcagga	gaaatgcaag	caagagatgg	7500
	caagccagtg	agttaagcat	tctgtgtggg	gagcaggtgg	atgaagagag	aggctgggct	7560
20		gggggggt					7620
20		atttttcctg					7680
		ccaccattgc					7740
		cggaaacaga					7800
		ttgtgtgtat					7860
25		ctgcaggaag					7920
25		gagaggagag					7980
	gcattgttgg	ggtgtgtgtg	tgtgtgtt	gtttatattt	gtattggaaa	tacattcttt	8040
	taaaaaatac	ttatccattt	atttatttt	atgtgcacgt	gtgtgtgcct	gcatgagttc	8100
		cgtgtgtgcg					8160
20	aactggagtt	ggaggaggtt	gtgagtcccc	tgacatgttt	gctgggaact	gaaccccggt	8220
30		agcaggaagt					8280
		atacacgtgg					8340
		tccggcacag					8400
		cacccccatg					8460
25	cacctcttgc	tctgcctctg	tcgctggaga	acagtgtgca	tctgcacact	cttatgtcag	8520
35		cagcctgcac					8580
	acacactact	gtactgcatt	ctctcgctct	Cttttttaa	acatatttt	atttgtttgt	8640

	gtgtatgcac	atgtgccaca	tgtgtacaga	tactatggag	gccagaagag	gccatggccg	8700
	tccctggagc	tggagttaca	ggcagcgtgt	gagctgcctg	gtgtgggtgc	tgggaaccaa	8760
	acttgaatct	aaagcaagca	cttttaactg	ctgaggcagc	tctcagtacc	cttcttcatt	8820
	tctccgcctg	ggttccattg	tatggacaca	tgtagctaga	atatcttgct	tatctaatta	8880
5	tgtacattgt	tttgtgctaa	gagagagtaa	tgctctatag	cctgagctgg	cctcaacctt	8940
	gccatcctcc	tgcctcagcc	tcctcctcct	gagtgctagg	atgacaggcg	agtggtaact	9000
	tacatggttt	catgttttgt	tcaagactga	aggataacat	tcatacagag	aaggtctggg	9060
	tcacaaagtg	tgcagttcac	tgaatggcac	aacccgtgat	caagaaacaa	aactcagggg	9120
	ctggagagat	ggcactgact	gctcttccag	aggtccggag	ttcaattccc	agcaaccaca	9180
10	tggtggctca	cagccatcta	taacgagatc	tgacgccctc	ttctggtgtg	tctgaagaca	9240
	gctacagtgt	actcacataa	aataaataaa	tctttaaaac	acacacacac	acacaattac	9300
	caccccagaa	agcccactcc	atgttccctc	ccacgtetet	gcctacagta	ctcccaggtt	9360
	accactgttc	aggcttctaa	caacctggtt	tacttgggcc	tctttctgc	tctgtggagc	9420
	cacacatttg	tgtgcctcat	acacgttctt	tctagtaagt	tgcatattac	tctgcgtttt	9480
15	tacatgtatt	tatttattgt	agttgtgtgt	gcgtgtgggc	ccatgcatgg	cacagtgtgt	9540
	ggggatgtca	gagtattgtg	aacaggggac	agttcttttc	ttcaatcatg	tgggttccag	9600
	aggttgaact	caggtcatca	tgtgtggcag	caaatgcctt	tacccactga	gacateteca :	9660
	tattctttt	ttttcccctg	aggtgggggc	ttgttccata	gcccaaactg	gctttgcact	9720
					ttggaattac		9780
20	accacacctg	actggatcat	taattctttg	atgggggcgg	ggaagcgcac	atgctgcagg	9840
					gaacagcttc		9900
	tctcccaact	gagctatttc	ggtttgccag	agaacaactt	acagaaagtt	ctcagtgcca	9960
					tggctcctct		10020
					aaaaagactt		10080
25					ccagaggtcc		10140
					tcaacgtgag		10200
					cctggtcatt		10260
					acagtgaagt		10320
					tgtccattgc		10380
30					gcggcagaag		10440
	agtctgctct	gagtactgtg	aggcagaatc	gtgagatcgg	ccccaacgat	ggcttcctgg	10500
	cccaactctg	ccagctcaat	gacagactag	ccaaggaggg	caaggtgaaa	ctctagggtg	10560
	cccacagcct	cttttgcaga	ggtctgactg	ggagggccct	ggcagccatg	tttaggaaac	10620
	acagtatacc	cactccctgc	accaccagac	acgtgcccac	atctgtccca	ctctggtcct	10680
35					cctaagaagt		10740
	agccatcctt	tcctgtaatt	tatgtctctc	cctgaggtga	ggttcaggtt	tatgtccctg	10800

	tctgtggcat	t agatacatct	cagtgaccca	gggtgggagg	gctatcaggg	g tgcatggccc	10860
	gggacacggg	g cactcttcat	gacccctccc	ccacctgggt	tetteetgte	g tggtccagaa	10920
	ccacgagcct	ggtaaaggaa	ctatgcaaac	acaggccctg	acctccccat	gtctgttcct	10980
	ggtcctcaca	gcccgacacg	ccctgctgag	gcagacgaat	gacattaagt	tctgaagcag	11040
5	agtggagata	gattagtgac	tagatttcca	aaaagaagga	aaaaaaaggc	tgcattttaa	11100
	aattatttco	: ttagaattaa	agatactaca	taggggccct	tgggtaagca	aatccatttty	11160
	tcccagaggc	tatcttgatt	ctttggaatg	tttaaagtgt	gccttgccag	agagcttacg	11220
	atctatatct	gctgcttcag	agccttccct	gaggatggct	ctgttccttt	gcttgttaga	11280
	agagcgatgc	cttgggcagg	gtttccccct	tttcagaata	cagggtgtaa	agtccagcct	11340
10	attacaaaca	aacaaacaaa	caaacaaaca	aaggacctcc	atttggagaa	ttgcaaggat	11400
	tttatcctga	attatagtgt	tggtgagttc	aagtcatcac	gccaagtgct	tgccatcctg	11460
	gttgctattc	taagaataat	taggaggagg	aacctagcca	attgcagctc	atgtccgtgg	11520
	gtgtgtgcac	gggtgcatat	gttggaaggg	gtgcctgtcc	ccttggggac	agaaggaaaa	11580
	tgaaaggccc	ctctgctcac	cctggccatt	tacgggaggc	tctgctggtt	ccacggtgtc	11640
15	tgtgcaggat	cctgaaactg	actcgctgga	cagaaacgag	acttggcggc	accatgagaa	11700
	tggagagaga	gagagcaaag	aaagaaacag	cctttaaaag	aactttctaa	gggtggtttt	11760
	tgaacctcgc	tggaccttgt	atgtgtgcac	atttgccaga	gattgaacat	aatcctcttg	11820
	ggacttcacg	ttctcattat	ttgtatgtct	ccggggtcac	gcagagccgt	cagccaccac	11880
20	cccagcaccc	ggcacatagg	cgtctcataa	aagcccattt	tatgagaacc	agagctgttt	11940
20	gagtaccccg	tgtatagaga	gagttgttgt	cgtggggcac	ccggatccca	gcagcctggt	12000
	tgcctgcctg	taggatgtct	tacaggagtt	tgcagagaaa	ccttccttgg	agggaaagaa	12060
	atatcaggga	tttttgttga	atatttcaaa	ttcagcttta	agtgtaagac	tcagcagtgt	12120
	tcatggttaa	ggtaaggaac	atgccttttc	cagagetget	gcaagaggca	ggagaagcag	12180
25	acctgtctta	ggatgtcact	cccagggtaa	agacctctga	tcacagcagg	agcagagctg	12240
25	tgcagcctgg	atggtcattg	tcccctattc	tgtgtgacca	cagcaaccct	ggtcacatag	12300
	ggctggtcat	ccttttttt	ttttttttt	ttttttttg	gcccagaatg	aagtgaccat	12360
	agccaagttg	tgtacctcag	tctttagttt	ccaagcggct	ctcttgctca	atacaatgtg	12420
	catttcaaaa	taacactgta	gagttgacag	aactggttca	tgtgttatga	gagaggaaaa	12480
20	gagaggaaag	aacaaacaa	aacaaaacac	cacaaaccaa	aaacatctgg	gctagccagg	12540
30	catgattgca	atgtctacag	gcccagttca	tgagaggcag	agacaggaag	accgccgaaa	12600
	ggtcaaggat	agcatggtct	acgtatcgag	actccagcca	gggctacggt	cccaagatcc	12660
	taggttttgg	attttgggct	ttggtttttg	agacagggtt	tctctgtgta	gccctggctg	12720
	ccctggaact	cgctctgtag	accaggctgg	cctcaaactt	agagatctgc	ctgactctgc	12780
35	artes	tgggacgaat	gccaccactg	cccaactaag	attccattaa	aaaaaaaaa	12840
ر د	agttcaagat	aattaagagt	tgccagctcg	ttaaagctaa	gtagaagcag	tctcaggcct	12900
	gctgcttgag	gctgttcttg	gcttggacct	gaaatctgcc	cccaacagtg	tccaagtgca	12960

	catgactttg	agccatctcc	agagaaggaa	gtgaaaattg	tggctcccca	gtcgattggg	13020
	acacagtctc	tctttgtcta	ggtaacacat	ggtgacacat	agcattgaac	tetecaetet	13080
						acagccacag	13140
	gacagtcact	agcacctact	ggaaacctct	ttgtgggaac	atgaagaaag	agcctttggg	13200
5		ctttccatta					13260
	gtttataaaa	ctagctacta	ttcttcaggt	aaaataccga	tgttgtggaa	aagccaaccc	13320
	cgtggctgcc	cgtgagtagg	gggtggggtt	gggaatcctg	gatagtgttc	tatccatgga	13380
	aagtggtgga	ataggaatta	agggtgttcc	cccccccc	aacctcttcc	tcagacccag	13440
	ccactttcta	tgacttataa	acatccaggt	aaaaattaca	aacataaaaa	tggtttctct	13500
10	tctcaatctt	ctaaagtctg	cctgcctttt	ccaggggtag	gtctgtttct	ttgctgttct	13560
	attgtcttga	gagcacagac	taacacttac	caaatgaggg	aactcttggc	ccatactaag	13620
		gctccagcac					13680
		cccaagtggg					13740
	gggtgccttt	ccaccttaag	ttgcttatag	tatttaagat	gctaaatgtt	ttaatcaaga	13800
15		tcttataata					13860
		acaggctttg					13920
		tttcctctgc					13980
		agagggcagg					14040
		tgccatccgg					14100
20		taaagttgta					14160
		tgtgtgtttg					14220
		tgaggtagga					14280
		cccctgtccc					14340
		tttttctaac					14400
25		cacactgctc					14460
		gtccagagga					14520
		acagatacac					14580
		gaggtacgac					14640
	ttctggctaa						14700
30	atggaggaag						14760
	aggacactag						14820
	ttatgctggg						14880
	attttttaa						14940
	ctgagagctg						15000
35	ggctgtggtg						15060
	gctagcctgt	ggtgcatgag	accctgtttc	aaaaacttta	ataaagaaat	aatgaaaaaa	15120

	gacatcaggg	cagateettg	gggccaaagg	g cggacaggcg	agtctcgtgg	taaggtcgtg	15180
	tagaagcgga	tgcatgagca	cgtgccgcag	g gcatcatgag	agagecetag	gtaagtaagg	15240
	atggatgtga	gtgtgtcggc	gtcggcgcac	tgcacgtcct	ggctgtggtg	ctggactggc	15300
						tccgaattat	15360
5	ttcaagaact	gtctattaca	attatctcaa	aatattaaaa	aaaaagaaga	attaaaaaac	15420
	aaaaaaccta	tccaggtgtg	gtggtgtgca	cctatagcca	cgggcacttg	gaaagctgga	15480
	gcaagaggat	ggcgagtttg	aaggtatctg	gggctgtaca	gcaagaccgt	cgtccccaaa	15540
	ccaaaccaaa	cagcaaaccc	attatgtcac	acaagagtgt	ttatagtgag	cggcctcgct	15600
••	gagagcatgg	ggtggggtg	ggggtggggg	acagaaatat	ctaaactgca	gtcaataggg	15660
10	atccactgag	accctggggc	ttgactgcag	cttaaccttg	ggaaatgata	agggttttgt	15720
	gttgagtaaa	agcatcgatt	actgacttaa	cctcaaatga	agaaaaagaa	aaaaagaaaa	15780
	caacaaaagc	caaaccaagg	ggctggtgag	atggctcagt	gggtaagagc	accegactge	15840
	tcttccgaag	gtccagagtt	caaatcccag	caaccacatg	gtggctcaca	accatctgta	15900
	acgagatatg	atgccctctt	ctggtgtgtc	tgaagacagc	tacagtgtac	ttacatataa	15960
15	taaataaatc	ttaaaaaaaa	aaaaaaaaa	aaaagccaaa	ccgagcaaac	caggccccca	16020
	aacagaaggc	aggcacgacg	gcaggcacca	cgagccatcc	tgtgaaaagg	cagggctacc	16080
	catgggccga	ggagggtcca	gagagatagg	ctggtaagct	cagtttctct	gtataccctt	16140
	tttcttgttg	acactacttc	aattacagat	aaaataacaa	ataaacaaaa	tctagagcct	16200
20	ggccactctc	tgctcgcttg	atttttcctg	ttacgtccag	caggtggcgg	aagtgttcca	16260
20	aggacagatc	gcatcattaa	ggtggccagc	ataatctccc	atcagcaggt	ggtgctgtga	16320
	gaaccattat	ggtgctcaca	gaatcccggg	cccaggagct	gccctctccc	aagtctggag	16380
	caataggaaa	gctttctggc	ccagacaggg	ttaacagtcc	acattccaga	gcaggggaaa	16440
	aggagactgg	aggtcacaga	caaaagggcc	agcttctaac	aacttcacag	ctctggtagg	16500
25	agagatagat	cacccccaac	aatggccaca	gctggttttg	tctgccccga	aggaaactga	16560
25	cttaggaagc	aggtatcaga	gtccccttcc	tgaggggact	tctgtctgcc	ttgtaaagct	16620
	gtcagagcag	ctgcattgat	gtgtgggtga	cagaagatga	aaaggaggac	ccaggcagat	16680
	cgccacagat	ggaccggcca	cttacaagtc	gaggcaggtg	gcagagcctt	gcagaagctc	16740
	tgcaggtgga	cgacactgat	tcattaccca	gttagcatac	cacageggge	taggcggacc	16800
20	acageeteet	tcccagtctt	cctccagggc	tggggagtcc	tccaaccttc	tgtctcagtg	16860
30	cagcttccgc	cagcccctcc	tccttttgca	cctcaggtgt	gaaccctccc	tcctctcctt	16920
	ctccctgtgg	catggccctc	ctgctactgc	aggctgagca	ttggatttct	ttgtgcttag	16980
	atagacctga	gatggctttc	tgatttatat	atatatatcc	atcccttgga	tcttacatct	17040
	aggacccaga	gctgtttgtg	ataccataag	aggctgggga	gatgatatgg	taagagtgct	17100
2.5	tgctgtacaa	gcatgaagac	atgagttcga	atccccagca	accatgtgga	aaaataacct	17160
35	tctaacctca	gagttgaggg	gaaaggcagg	tggattctgg	gggcttactg	gccagctagc	17220
	cagcctaacc	taaatgtctc	agtcagagat	cctgtctcag	ggaataactt	gggagaatga	17280

	ctgagaaaga	cacctcctca	ggtctcccat	gcacccacac	agacacacgg	gggggggta	17340
	atgtaataag	ctaagaaata	atgagggaaa	tgatttttg	ctaagaaatg	aaattctgtg	17400
	ttggccgcaa	gaagcctggc	cagggaagga	actgcctttg	gcacaccago	ctataagtca	17460
	ccatgagttc	cctggctaag	aatcacatgt	aatggagccc	aggtccctct	tgcctggtgg	17520
5	ttgcctctcc	cactggtttt	gaagagaaat	tcaagagaga	tctccttggt	cagaattgta	17580
	ggtgctgagc	aatgtggagc	tggggtcaat	gggattcctt	taaaggcatc	cttcccaggg	17640
	ctgggtcata	cttcaatagt	agggtgcttg	cacagcaagc	gtgagaccct	aggttagagt	17700
	ccccagaatc	tgcccccaac	ccccaaaaa	ggcatccttc	tgcctctggg	tgggtggggg	17760
	gagcaaacac	ctttaactaa	gaccattagc	tggcaggggt	aacaaatgac	cttggctaga	17820
10	ggaatttggt	caagctggat	tccgccttct	gtagaagccc	cacttgtttc	ctttgttaag	17880
	ctggcccaca	gtttgttttg	agaatgcctg	aggggcccag	ggagccagac	aattaaaagc	17940
	caagctcatt	ttgatatctg	aaaaccaçag	cctgactgcc	ctgcccgtgg	gaggtactgg	18000
	gagagctggc	tgtgtccctg	cctcaccaac	gcccccccc	ccaacacaca	ctcctcgggt	18060
	cacctgggag	gtgccagcag	caatttggaa	gtttactgag	cttgagaagt	cttgggaggg	18120
15	ctgacgctaa	gcacacccct	tctccacccc	ccccacccc	acccccgtga	ggaggagggt	18180
	gaggaaacat	gggaccagcc	ctgctccagc	ccgtccttat	tggctggcat	gaggcagagg	18240
	gggctttaaa	aaggcaaccg	tatctaggct	ggacactgga	gcctgtgcta	ccgagtgccc	18300
	tcctccacct	ggcagcatgc	agccctcact	agccccgtgc	ctcatctgcc	tacttgtgca	18360
	cgctgccttc	tgtgctgtgg	agggccaggg	gtggcaagcc	ttcaggaatg	atgccacaga	18420
20	ggtcatccca	gggcttggag	agtaccccga	gcctcctcct	gagaacaacc	agaccatgaa	18480
	ccgggcggag	aatggaggca	gacctcccca	ccatccctat	gacgccaaag	gtacgggatg	18540
	aagaagcaca	ttagtggggg	ggggggtcct	gggaggtgac	tggggtggtt	ttagcatctt	18600
	cttcagaggt	ttgtgtgggt	ggctagcctc	tgctacatca	gggcagggac	acatttgcct	18660
	ggaagaatac	tagcacagca	ttagaacctg	gagggcagca	ttggggggct	ggtagagagc	18720
25	acccaaggca	gggtggaggc	tgaggtcagc	cgaagctggc	attaacacgg	gcatgggctt	18780
	gtatgatggt	ccagagaatc	tcctcctaag	gatgaggaca	caggtcagat	ctagctgctg	18840
	accagtgggg	aagtgatatg	gtgaggctgg	atgccagatg	ccatccatgg	ctgtactata	18900
	tcccacatga	ccaccacatg	aggtaaagaa	ggccccagct	tgaagatgga	gaaaccgaga	18960
	ggctcctgag	ataaagtcac	ctgggagtaa	gaagagctga	gactggaagc	tggtttgatc	19020
30	cagatgcaag	gcaaccctag	attgggtttg	ggtgggaacc	tgaagccagg	aggaatccct	19080
	ttagttcccc	cttgcccagg	gtctgctcaa	tgagcccaga	gggttagcat	taaaagaaca	19140
				cagctgagtg			19200
				cctgacccca			19260
				cagagagatg			19320
35				ggtggggtgg			19380
				gggaggagag			19440

	cacatgctg	t ccctcctgtc	tcctagccag	g taagggatg	t ggaggaaagg	g gccaccccaa	19500
	aggagcatg	c aatgcagtca	cgtttttgca	a gaggaagtg	c ttgacctaac	ggcactattc	19560
	ttggaaagc	c ccaaaactag	teetteeet	ggcaaacag	g cctccccad	ataccacctc	19620
	tgcaggggt	g agtaaattaa	gccagccaca	a gaagggtgg	aaggcctaca	cctccccct	19680
5	gttgtgccc	ccccccccc	gtgaaggtgo	atcctggcct	ctgcccctct	ggctttggta	19740
	ctgggattt	ttttttcctt	ttatgtcata	ttgatcctga	a caccatggaa	Cttttggagg	19800
	tagacaggad	ccacacatgg	attagttaaa	agcctcccat	ccatctaago	tcatggtagg	19860
	agatagagca	tgtccaagag	aggagggcag	gcatcagaco	tagaagatat	ggctgggcat	19920
	ccaacccaat	ctccttcccc	ggagaacaga	ctctaagtca	gatccagcca	cccttgagta	19980
10	accagctcaa	ggtacacaga	acaagagagt	ctggtataca	gcaggtgcta	aacaaatgct	20040
	tgtggtagca	aaagctatag	gttttgggtc	agaactccga	cccaagtcgc	gagtgaagag	20100
	cgaaaggccc	tctactcgcc	accgccccgc	ccccacctgg	ggicctataa	cagatcactt	20160
	tcacccttgc	gggagccaga	gagccctggc	atcctaggta	gcccccccg	cccccccc	20220
	gcaagcagcc	cagecetgee	tttggggcaa	gttcttttct	cagcctggac	Ctgtgataat	20280
15	gaggggttg	gacgcgccgc	ctttggtcgc	tttcaagtct	aatgaattct	tatccctacc	20340
	acctgccctt	ctaccccgct	cctccacagc	agctgtcctg	atttattacc	ttcaattaac	20400
	ctccactcct	ttctccatct	cctgggatac	cgcccctgtc	ccagtggctg	gtaaaggagc	20460
	ttaggaagga	ccagagccag	gtgtggctag	aggctaccag	gcagggctgg	ggatgaggag	20520
20	Ctaaactgga	agagtgtttg	gttagtaggc	acaaagcctt	gggtgggatc	cctagtaccg	20580
20	gagaagtgga	gatgggcgct	gagaagttca	agaccatcca	tccttaacta	cacagccagt	20640
	ttgaggccag	cctgggctac	ataaaaaccc	aatctcaaaa	gctgccaatt	ctgattctgt	20700
	gccacgtagt	gcccgatgta	atagtggatg	aagtcgttga	atcctggggc	aacctatttt	20760
	acagatgtgg	ggaaaagcaa	ctttaagtac	cctgcccaca	gatcacaaag	aaagtaagtg	20820
25	acagagetee	agtgtttcat	ccctgggttc	caaggacagg	gagagagaag	ccagggtggg	20880
25 .	acctcactge	tccccggtgc	ctccttccta	taatccatac	agattcgaaa	gcgcagggca	20940
	ggcccggaaa	aagagagaag	ggtggaagga	gcagaccagt	ctggcctagg	ctgcagcccc	21000
	cattanta	ctctctccgc	agatgtgtcc	gagtacagct	gccgcgagct	gcactacacc	21060
	cgctteetga	cagacggccc	atgccgcagc	gccaagccgg	tcaccgagtt	ggtgtgctcc	21120
30	ggccagtgcg	gccccgcgcg	gctgctgccc	aacgccatcg	ggcgcgtgaa	gtggtggcgc	21180
30	ctgaacggac	cggatttccg	ctgcatcccg	gatcgctacc	gcgcgcagcg	ggtgcagctg	21240
	tagaagaaga	ggggcgcggc	gccgcgctcg	cgcaaggtgc	gtctggtggc	ctcgtgcaag	21300
	cgcaagegee	tcacccgctt	ccacaaccag	tcggagctca	aggacttcgg	gccggagacc	21360
	gegeggeege	agaagggtcg	caagccgcgg	cccggcgccc	ggggagccaa	agccaaccag	21420
35	attootete	agaacgccta	ctagagcgag	cccgcgccta	tgcagccccc	gcgcgatccg	21480
<i>.</i>	tagageeas	agtgtaaagc	ctgcagccca	ggccaggggt	gccaaacttt	ccagaccgtg	21540
	cyyayıtıdı	agcccagtag a	agaccgcagg	tccttctgcc	cgctgcgggg	gatggggagg	21600

	gggtggggt	cccgcgggcc	aggagagga	a gcttgagtco	cagactctgo	ctagccccgg	21660
	gtgggatgg	g ggtctttcta	ccctcgccg	g acctataca	gacaaggcag	tgtttccacc	21720
	ttaaaggga	a gggagtgtgg	aacgaaaga	ctgggactg	ttatggacgt	acagtaagat	21780
	ctactcctt	c cacccaaatg	taaagcctgo	gtgggctaga	tagggtttct	gaccctgacc	21840
5	tggccactga	gtgtgatgtt	gggctacgt	gttctcttt	ggtacggtct	tctttgtaaa	21900
	atagggaccg	gaactctgct	gagattccaa	a ggattggggt	accccgtgta	gactggtgag	21960
	agagaggaga	a acaggggagg	ggttagggga	a gagattgtgg	tgggcaaccg	cctagaagaa	22020
	gctgtttgtt	ggctcccagc	ctcgccgcct	cagaggtttg	gcttccccca	ctccttcctc	22080
	tcaaatctgo	cttcaaatcc	atatctggga	tagggaaggc	cagggtccga	gagatggtgg	22140
10	aagggccaga	aatcacactc	ctggccccc	gaagagcagt	gtcccgcccc	caactgcctt	22200
	gtcatattgt	aaagggattt	tctacacaac	agtttaaggt	cgttggagga	aactgggctt	22260
	gccagtcacc	tcccatcctt	gtcccttgcc	aggacaccac	ctcctgcctg	ccacccacgg	22320
	acacatttct	gtctagaaac	agagcgtcgt	cgtgctgtcc	tctgagacag	catatcttac	22380
	attaaaaaga	ataatacggg	aaaaaaaaac	ggagggcgca	agtgttatac	atatgctgag	22440
15	aagctgtcag	gcgccacagc	accacccaca	atctttttgt	aaatcatttc	cagacacctc	22500
	ttactttctg	tgtagatttt	aattgttaaa	aggggaggag	agagagcgtt	tgtaacagaa	22560
	gcacatggag	ggggggtag	gggggttggg	gctggtgagt	ttggcgaact	ttccatgtga	22620
	gactcatcca	caaagactga	aagccgcgtt	tttttttta	agagttcagt	gacatattta	22680
20	ttttctcatt	taagttattt	atgccaacat	ttttttcttg	tagagaaagg	cagtgttaat	22740
20	atcgctttgt	gaagcacaag	tgtgtgtggt	tttttgttt	ttgtttttc	cccgaccaga	22800
	ggcattgtta	ataaagacaa	tgaatctcga	gcaggaggct	gtggtcttgt	tttgtcaacc	22860
	acacacaatg	tctcgccact	gtcatctcac	tcccttccct	tggtcacaag	acccaaacct	22920
	tgacaacacc	tccgactgct	ctctggtagc	ccttgtggca	atacgtgttt	cctttgaaaa	22980
25	greacattea	tcctttcctt	tgcaaacctg	gctctcattc	cccagctggg	tcatcgtcat	23040
25	accctcaccc	cagcctccct	ttagctgacc	actctccaca	ctgtcttcca	aaagtgcacg	23100
	teteacegag	ccagttccct	ggtccaggtc	atcccattgc	tcctccttgc	tccagaccct	23160
	teteccacaa	agatgttcat	ctcccactcc	atcaagcccc	agtggccctg	cggctatccc	23220
	agetter	gttagctgaa	tctacttgct	gacaccacat	gaattccttc	ccctgtctta	23280
30	aggittatgg	aactcttgcc	tgcccctgaa	ccttccagga	ctgtcccagc	gtctgatgtg	23340
.,0	testteste	tgtaaagccc	caccccacta	tttgattccc	aattctagat	cttcccttgt	23400
	CCACCCCCEC	acgggatagt	gtctcatctg	gccaagtcct	gcttgatatt	gggataaatg	23460
	ttttagage	tacaattgag	gaccagttca	tcattgggcc	aagctttttc	aaaatgtgaa	23520
	Caacatcae	atagaagtgt	aaaagccttc	caaagcagag	gcaatgcctg	gctcttcctt	23580
35	aggggtata	gctcctgctt	tatgggtctg	gtggggtagt	acattcataa	acccaacact	23640
JJ	tcaaccteta	aagcaagatg	attgggagtt	cgaggccaat	cttggctatg	aggccctgtc	23700
	CCAACCLCLC	ctccctccct	ccagggtttt	gttttgtttt	gtttttttga	tttgaaactg	23760

						cctaatataa	23820
	gcttccatct	tgatttgtgt	atgtgcacac	tgggggttga	acctgggcct	ttgtacctgc	23880
						caactcccaa	23940
						gagtgctgct	24000
5						catcctgaag	24060
	aatgtcttag	agcaggaggc	catggagacc	ttggccagcc	ccacaaggca	gtgtggtgca	24120
	gagggtgagg	atggaggcag	gcttgcaatt	gaagctgaga	cagggtactc	aggattaaaa	24180
		aaaacaattc					24240
	gagcccagtg	ggcataggtg	aagacaccgg	ttgtactgtc	atgtactaac	tgtgcttcag	24300
10	agccggcaga	gacaaataat	gttatggtga	ccccagggga	cagtgattcc	agaaggaaca	24360
	cagaagagag	tgctgctaga	ggctgcctga	aggagaaggg	gtcccagact	ctctaagcaa	24420
	agactccact	cacataaaga	cacaggetga	gcagagctgg	ccgtggatgc	agggagccca	24480
	tccaccatcc	tttagcatgc	ccttgtattc	ccatcacatg	ccagggatga	ggggcatcag	24540
	agagtccaag	tgatgcccaa	acccaaacac	acctaggact	tgctttctgg	gacagacaga	24600
15		actaggttgg					24660
		aacaaaaaaa					24720
		gagtttattt					24780
		agaaagaaca					24840
		tgcttggggt					24900
20		gagagtcaag					24960
		cacacacaca					25020
		agaacaaacc					25080
		ggtccccact					25140
	gggtctgcag	aggcttcctg	ggtgacccag	agccacagac	actgaaatct	ggtgctgaga	25200
25		ccctcttcca					25260
		gaggatgaga					25320
		gggcacatga					25380
		gtttgtgatt					25440
		tacaagcctg					25500
30		cagacagtta					25560
		agacagccag					25620
		caggagacaa					25680
		gactagggca					25740
35		agtcttattc					25800
		tggctgaggt					25860
	ttggggaagc	tccctgcctg	cctgtaaatg	tgtccattct	tcaaccttag	acaagatcac	25920

	tttccctgag	cagtcaggcc	agtccaaagc	ccttcaattt	agctttcata	aggaacaccc	25980
	cttttgttgg	gtggaggtag	cacttgcctt	gaatcccagc	attaagaagg	cagagacagt	26040
	cggatctctg	tgagttcaca	gccagcctgg	tctacggagt	gagttccaag	acagccaggc	26100
	ctacacagag	aaaccctgtc	tcgaaaaaaa	caaaaacaaa	agaaataaag	aaaaagaaaa	26160
5	caaaaacgaa	caaacagaaa	aacaagccag	agtgtttgtc	cccgtatttt	attaatcata	26220
	tttttgtccc	tttgccattt	tagactaaaa	gactcgggaa	agcaggtctc	tctctgtttc	26280
	tcatccggac	acacccagaa	ccagatgtat	ggaagatggc	taatgtgctg	cagttgcaca	26340
	tctggggctg	ggtggattgg	ttagatggca	tgggctgggt	gtggttacga	tgactgcagg	26400
	agcaaggagt	atgtggtgca	tagcaaacga	ggaagtttgc	acagaacaac	actgtgtgta	26460
10	ctgatgtgca	ggtatgggca	catgcaagca	gaagccaagg	gacagcctta	gggtagtgtt	26520
	tccacagacc	cctccccct	tttaacatgg	gcatctctca	ttggcctgga	gcttgccaac	26580
	tgggctgggc	tggctagctt	gtaggtccca	gggatctgca	tatctctgcc	tecetagtge	26640
	tgggattaca	gtcatatatg	agcacacctg	gcttttttat	gtgggttctg	ggctttgaac	26700
	ccagatctga	gtgcttgcaa	ggcaatcggt	tgaatgactg	cttcatctcc	ccagaccctg	26760
15	ggattctact	ttctattaaa	gtatttctat	taaatcaatg	agcccctgcc	cctgcactca	26820
	gcagttctta	ggcctgctga	gagtcaagtg	gggagtgaga	gcaagcctcg	agaccccatc	26880
					ggctcgggat		26940
					caggaagggt		27000
					gcattgcaat		27060
20					taggtggtgt		27120
					tgcgccgcaa		27180
•					gaagggagct		27240
					gagacttgac		27300
					agcttcgtcc		27360
25					gcaagaaaga		27420
					tgcctgacaa		27480
					atggtcaggg		27540
					cttgtgctct		27600
••					tcggctgatc		27660 .
30					atggtgcatg		27720
					gggataaagc		27780
					tggtttttcg		27840
					accaggctgg		27900
					aggcctgcgc		27960
35					tattataatt		28020
	gttcattgct	gtagaattgg	agtcttcata	ttccaggtaa	tctcccacag	acatgccaca	28080

	aaacaacctg	, ttctacgaaa	tctctcatgg	actcccttcc	ccagtaatto	taaactgtgt	28140
	caaatctaca	agaaatagtg	acagtcacag	tctctaacgt	tttgggcatg	agtctgaagt	28200
						ggagcagagc	28260
						agaggggcc	28320
5		ccatacacct					28380
	ctagcaagaa	ggccttccct	ggatcaccca	ccaccttgca	cctccagaac	tcagagccaa	28440
	attaaacttt	cttgttactg	tcgtcaaagc	acagtcggtc	tgggttgtat	cactgtcaat	28500
						aaatgaaaag	28560
		aaaaagaaaa					28620
10	ttgtcctccc	cgaggaggtg	gcgagtaagg	tgtaaatgtt	catggatgta	aatgggccca	28680
	tatatgaggg	tctggggtaa	caagaaggcc	tgtgaatata	aagcactgaa	ggtatgtcta	28740
		ggtcactaca					28800
		cacacacaca					28860
		agtacttaaa					28920
15		tgagttgaaa					28980
		acgtttttct					29040
		tacatatgct					29100
		catctgtggc					29160
		tggggtggaa					29220
20		tgtggctcca					29280
		cacttagttc					29340
		gctttggctc					29400
		ttccacatgg					29460
26		cagtgtctct					29520
25		ctgggagttc					29580
	gggcaatgtt	gggaaaacct	ttctcaaaca	aaaagagggg	ttcagttgtc	aggaggagac	29640
	ccatgggtta	agaagtctag	acgagccatg	gtgatgcata	cctttcatcc	aagcacttag	29700
	gaggcaaaga	aaggtgaaac	tctttgactt	tgaggccagc	taggttacat	agtgataccc	29760
	tgcttagtgt	gtgtgtgtgt	gtgtgtgtgt	gtgtgtgtgt	gtgtgtaatt	taaaagtcta	29820
30	aaaatgcatt	Cttttaaaaa	tatgtataag	tatttgcctg	cacatatgta	tgtatgtatg	29880
	tataccatgt	gtgtgtctgg	tgctgaagga	ctaggcatag	actccctaga	actagagtca	29940
	tagacagttg	tgacactccc	caacccccca	ccatgtgggt	gcttgaagct	aaactcctgt	30000
	cctttgtaaa	gcagcaggtg	tctatgaacc	ctgaaccatc	tctccagtct	ccagatgtgc	30060
35	attctcaaag	aggagtcctt	catatttccc	taaactgaac	atccttatca	gtgagcatcc	30120
		aaagctactg					30180
	gctcatgaaa	cttaagtaca	cacacacaaa	cacacacaca	cacacagagt	catgcactca	30240

	caaaagcatg	catgtacacc	attcttatta	gactatgctt	tgctaaaaga	ctttcctaga	30300
	tactttaaaa	catcacttct	gccttttggt	gggcaggttc	caagattggt	actggcgtac	30360
	tggaaactga	acaaggtaga	gatctagaaa	tcacagcagg	tcagaagggc	cagcctgtac	30420
	aagagagagt	tccacacctt	ccaggaacac	tgagcagggg	gctgggacct	tgcctctcag	30480
5	cccaagaaac	tagtgcgttt	cctgtatgca	tgcctctcag	agattccata	agatetgeet	30540
	tctgccataa	gatctcctgc	atccagacaa	gcctagggga	agttgagagg	ctgcctgagt	30600
	ctctcccaca	ggccccttct	tgcctggcag	tatttttta	tctggaggag	aggaatcagg	30660
	gtgggaatga	tcaaatacaa	ttatcaagga	aaaagtaaaa	aacatatata	tatatatatt	30720
	aactgatcta	gggagctggc	tcagcagtta	agagttctgg	ctgcccttgc	ttcagatctt	30780
10	gctttgattc	ccagcaccca	catgatggct	ttcaactgta	tctctgcttc	caggggatcc	30840
	aacagcctct	tctgacctcc	atagacaaga	cctagtcctc	tgcaagagca	ccaaatgctc	30900
	ttatctgttg	atccatctct	ctagcctcat	gccagatcat	ttaaaactac	tggacactgt	30960
	cccattttac	gaagatgtca	ctgcccagtc	atttgccatg	agtggatatt	tcgattcttt	31020
	ctatgttctc	acccttgcaa	tttataagaa	agatatctgc	atttgtctcc	tgagagaaca	31080
15			atggctctag				31140
	acttaagttt	ggtcttggaa	tccacatggt	ggagagagag	aagagattcc	cgtaagttgt	31200
			tgctgtggct				31260
			ggtttcttca				31320
			ccattgtgcc				31380
20			taatgagcta				31440
*	tggcggcgta	atgacctcca	ccatgatgtt	atccagcatg	aaggtcctca	ccagaagtca	31500
	tacaaatctt	cttaggcttc	cagagtcgtg	agcaaaaaaa	gcacacctct	aaataaatta	31560
	actagcctca	ggtagttaac	caccgaaaat	gaaccaaggc	agttctaata	caaaaccact	31620
	tcccttccct	gttcaaacca	cagtgcccta	ttatctaaaa	gataaacttc	aagccaagct	31680
25			gtaacaacaa				31740
	tactgggcct	caggggcaga	gacaggtgga	gccctggagt	ttgaattcca	ggttctgtga	31800
			aatatggtga				31860
	ctggccaaca	cacagccatc	tctgcacatc	tgtagttgca	agccttttgc	actaagtttg	31920
	gccagagtca	gagtttgcaa	gtgtttgtgg	actgaatgca	cgtgttgctg	gtgatctaca	31980
30	aagtcaccct	ccttctcaag	ctagcagcac	tggcttcggc	cagctgctca	ttcaagcctc	32040
	tttgcagagt	catcacgggg	atgggggagc	agggcccctc	cctagaacac	caagcctgtg	32100
	gttgtttatt						32160
	cagtcgggtg						32220
	aaaagaaata						32280
35	ccctggggaa						32340
	ctctaatctc						32400

	gtctgtgtac	tcacagggag	gagggtggca	aagccctggt	cctctacggg	ctgggggaag	32460
	gggggaagct	gtcggcccag	tgactttttc	ccctttctct	ttttcttaga	aaccagtctc	32520
	aatttaagat	aatgagtctc	ctcattcacg	tgtgctcact	attcataggg	acttatccac	32580
	ccccgccctg	tcaatctggc	taagtaagac	aagtcaaatt	taaaagggaa	cgtttttcta	32640
5	aaaatgtggc	tggaccgtgt	gccggcacga	aaccagggat	ggcggtctaa	gttacatgct	32700
	ctctgccagc	cccggtgcct	tttcctttcg	gaaaggagac	ccggaggtaa	aacgaagttg	32760
	ccaacttttg	atgatggtgt	gcgccgggtg	actctttaaa	atgtcatcca	tacctgggat	32820
	agggaaggct	cttcagggag	tcatctagcc	ctcccttcag	gaaaagattc	cacttccggt	32880
	ttagttagct	tccacctggt	cccttatccg	ctgtctctgc	ccactagtcc	tcatccatcc	32940
10	ggtttccgcc	ctcatccacc	ttgccctttt	agttcctaga	aagcagcacc	gtagtcttgg	33000
	caggtgggcc	attggtcact	ccgctaccac	tgttaccatg	gccaccaagg	tgtcatttaa	33060
	atatgagete	actgagtcct	gcgggatggc	ttggttggta	atatgcttgc	tgcaaaatcg	33120
	tgagaactgg	agttcaattc	ccagcacatg	gatgtatttc	cagcacctgg	aaggcaggga	33180
	gcagagatct	taaagctcct	ggccagacag	cccagcctaa	ttagtaatca	gtgagagacc	33240
15					ttgtctccac		33300
					acacacctga		33360
					tacatacacc		33420
					ggcttcaact		33480
					caaaggcttt		33540
20					cttttcttc		33600
					attcacccac		33660
					tgctgtaccc		33720
					ttcagcctag		33780
					aatctccttc		33840
25					gacctctaat		33900
					acctcagagt		33960
					ccggattctg		34020
					cagctgggtt		34080
					ggcagtggtg		34140
30					ggtggtggtg		34200
					gacaaatctg		34260
					ctttatgcca		34320
					acatggtatt		34380
					cccagtcctt		34440
35					cggagtgtgg		34500
	atggtctgga	cagaagcatc	cagagagggt	ccaagacaaa	tgcctcgcct	cctaaggaac	34560

	actggcagcc	ctgatgaggt	accagagatt	gctaagtgga	ggaatacagg	atcagaccca	34620
	tggaggggct	taaagcgtga	ctgtagcagc	cctccgctga	ggggctccag	gtgggcgccc	34680
	aaggtgctgc	agtgggagcc	acatgagagg	tgatgtcttg	gagtcacctc	gggtaccatt	34740
	ġtttagggag	gtggggattt	gtggtgtgga	gacaggcagc	ctcaaggatg	cttttcaaca	34800
5	atggttgatg	agttggaact	aaaacagggg	ccatcacact	ggctcccata	gctctgggct	34860
	tgccagcttc	cacatctgcc	ccccaccccc	tgtctggcac	cagctcaagc	tctgtgattc	34920
	tacacatcca	aaagaggaag	agtagcctac	tgggcatgcc	acctcttctg	gaccatcagg	34980
					ggctgccaga		35040
					atgcccgccc		35100
10					ttgatttatc		35160
					ttcatgtatc		35220
					ttcttttttg		35280
					actcactttg		35340
					caccacgccc		35400
15					acacacatgt		35460
					ccctggggcc		35520
					gatcaagtcc		35580
					gctccctggc		35640
					ctcaagttgt		35700
20					ctcaagcacg		35760
					gcgtgtggtt		35820
	acgtgtgc					-	35828

<210> 18

25 <211> 9301

<212> DNA

<213> Homo sapien

<400> 18

tagaggagaa gtctttgggg agggtttgct ctgagcacac ccctttccct ccctccgggg 30 60 ctgagggaaa catgggacca gccctgcccc agcctgtcct cattggctgg catgaagcag 120 agaggggctt taaaaaggcg accgtgtctc ggctggagac cagagcctgt gctactggaa 180 ggtggcgtgc cctcctctgg ctggtaccat gcagctccca ctggccctgt gtctcgtctg 240 cctgctggta cacacagcct tccgtgtagt ggagggccag gggtggcagg cgttcaagaa 300 35 tgatgccacg gaaatcatcc ccgagctcgg agagtacccc gagcctccac cggagctgga 360 gaacaacaag accatgaacc gggcggagaa cggagggcgg cctccccacc acccctttga 420

	gaccaaaggt	atggggtgga	ggagagaatt	cttagtaaaa	gatcctgggg	aggttttaga	480
	aacttctctt	tgggaggctt	ggaagactgg	ggtagaccca	gtgaagattg	ctggcctctg	540
	ccagcactgg	tcgaggaaca	gtcttgcctg	gaggtggggg	aagaatggct	cgctggtgca	600
	gccttcaaat	tcaggtgcag	aggcatgagg	caacagacgc	tggtgagagc	ccagggcagg	660
5	gaggacgctg	gggtggtgag	ggtatggcat	cagggcatca	gaacaggctc	aggggctcag	720
	aaaagaaaag	gtttcaaaga	atctcctcct	gggaatatag	gagccacgtc	cagctgctgg	780
	taccactggg	aagggaacaa	ggtaagggag	cctcccatcc	acagaacagc	acctgtgggg	840
	caccggacac	tctatgctgg	tggtggctgt	ccccaccaca	cagacccaca	tcatggaatc	900
	cccaggaggt	gaacccccag	ctcgaagggg	aagaaacagg	ttccaggcac	tcagtaactt	960
10	. ggtagtgaga	agagctgagg	tgtgaacctg	gtttgatcca	actgcaagat	agccctggtg	1020
	tgtgggggg	tgtgggggac	agatctccac	aaagcagtgg	ggaggaaggc	cagagaggca	1080
	cccctgcagt	gtgcattgcc	catggcctgc	ccagggagct	ggcacttgaa	ggaatgggag	1140
	ttttcggcac	agttttagcc	cctgacatgg	gtgcagctga	gtccaggccc	tggaggggag	1200
	agcagcatcc	tctgtgcagg	agtagggaca	tctgtcctca	gcagccaccc	cagtcccaac	1260
15	cttgcctcat	tccaggggag	ggagaaggaa	gaggaaccct	gggttcctgg	tcaggcctgc	1320
		ccaggtgaca					1380
		ctgaaatgac					1440
		gaagtccact					1500
		atggggtgct					1560
20		ggaggacaag					1620
		cgactgcctg					1680
		cgagaggcca					1740
		aagtgcctgg					1800
		aatgcccccc					1860
25		acagaccaga					1920
		cttcagagtg					1980
		atttttcatt					2040
		gtactcacac					2100
		acatggtcaa					2160
30		cagggagtat					2220
		gagagccagg					2280
		ccctatggta					2340
		cctcccatgg					2400
•		ccccagggaa					2460
35		ctgggcctca					2520
	tgattccttt	caagtctaat	gaattcctgt	cctgatcacc	tccccttcag	tccctcgcct	2580

	ccacagcago	tgccctgatt	tattaccttc	aattaacctc	tactcctttc	tccatcccct	2640
	gtccacccct	cccaagtggc	tggaaaagga	atttgggaga	agccagagcc	aggcagaagg	2700
						ggcttaaatc	2760
						gctttcagct	2820
5						caatgcttgg	2880
						aagatgacac	2940
						tccgagggga	3000
	cagggtgcgc	aggagagctt	tccaccagct	ctagagcatc	tgggaccttc	ctgcaataga	3060
						ggtggagaga	3120
10	gacgggccgg	tccagggcag	gggtggccag	gcgggcggcc	acceteaege	gcgcctctct	3180
	ccacagacgt	gtccgagtac	agctgccgcg	agctgcactt	cacccgctac	gtgaccgatg	3240
						tgcggcccgg	3300
			atcggccgcg				3360
	tccgctgcat	ccccgaccgc	taccgcgcgc	agcgcgtgca	gctgctgtgt	cccggtggtg	3420
15			gtgcgcctgg				3480
						ccgcagaagg	3540
						ctggagaacg	3600
			cccctcccca				3660
••			gcgtggtttg				3720
20			accttccagg				3780
			ggggtcccac				3840
			cctctggggc				3900
			tcaccgccct				3960
2.5			gaaagttgga				4020
25			cagagcacaa				4080
			ttgctgtgta				4140
			atgagggtgg				4200
			ccagtgcctt				4260
20			agttgcattg				4320
30			gacagccaaa				4380
			atatttacgg				4440
	ttcccagcct						4500
	catcatccat						4560
25	atccgcccca	acttcccaaa	gagcagcatc	cctcccccga	cccatagcca	tgttttaaag	4620
35	tcaccttccg	aagagaagtg	aaaggttcaa	ggacactggc	cttgcaggcc	cgagggagca	4680
	gccatcacaa	actcacagac	cagcacatcc	cttttgagac	accgccttct	gcccaccact	4740

	cacggacaca	tttctgccta	gaaaacagct	tcttactgct	cttacatgtg	atggcatatc	4800
	ttacactaaa	agaatattat	tgggggaaaa	actacaagtg	ctgtacatat	gctgagaaac	4860
	tgcagagcat	aatagctgcc	acccaaaaat	ctttttgaaa	atcatttcca	gacaacctct	4920
	tactttctgt	gtagttttta	attgttaaaa	aaaaaaagtt	ttaaacagaa	gcacatgaca	4980
5	tatgaaagcc	tgcaggactg	gtcgttttt	tggcaattct	tccacgtggg	acttgtccac	5040
	aagaatgaaa	gtagtggttt	ttaaagagtt	aagttacata	tttattttct	cacttaagtt	5100
	atttatgcaa	aagtttttct	tgtagagaat	gacaatgtta	atattgcttt	atgaattaac	5160
	agtctgttct	tccagagtcc	agagacattg	ttaataaaga	caatgaatca	tgaccgaaag	5220
	gatgtggtct	cattttgtca	accacacatg	acgtcatttc	tgtcaaagtt	gacacccttc	5280
10	tcttggtcac	tagagctcca	accttggaca	cacctttgac	tgctctctgg	tggcccttgt	5340
	ggcaattatg	tcttcctttg	aaaagtcatg	tttatccctt	cctttccaaa	cccagaccgc	5400
	atttcttcac	ccagggcatg	gtaataacct	cagccttgta	tccttttagc	agcctcccct	5460
	ccatgctggc	ttccaaaatg	ctgttctcat	tgtatcactc	ccctgctcaa	aagccttcca	5520
	tagctccccc	ttgcccagga	tcaagtgcag	tttccctatc	tgacatggga	ggccttctct	5580
15	gcttgactcc	cacctcccac	tccaccaagc	ttcctactga	ctccaaatgg	tcatgcagat	5640
	ccctgcttcc	ttagtttgcc	atccacactt	agcaccccca	ataactaatc	ctctttcttt	5700
	aggattcaca	ttacttgtca	tctcttcccc	taaccttcca	gagatgttcc	aatctcccat	5760
	gatccctctc	tcctctgagg	ttccagcccc	ttttgtctac	accactactt	tggttcctaa	5820
	ttctgttttc	catttgacag	tcattcatgg	aggaccagcc	tggccaagtc	ctgcttagta	5880
20	ctggcataga	caacacaaag	ccaagtacaa	ttcaggacca	gctcacagga	aacttcatct	5940
	tcttcgaagt	gtggatttga	tgcctcctgg	gtagaaatgt	aggatcttca	aaagtgggcc	6000
	agcctcctgc	acttctctca	aagtctcgcc	tccccaaggt	gtcttaatag	tgctggatgc	6060
	tagctgagtt	agcatcttca	gatgaagagt	aaccctaaag	ttactcttca	gttgccctaa	6120
	ggtgggatgg	tcaactggaa	agctttaaat	taagtccagc	ctaccttggg	ggaacccacc	6180
25	cccacaaaga	aagctgaggt	ccctcctgat	gacttgtcag	tttaactacc	aataacccac	6240
	ttgaattaat	catcatcatc	aagtctttga	taggtgtgag	tgggtatcag	tggccggtcc	6300
	cttcctgggg	ctccagcccc	cgaggaggcc	tcagtgagcc	cctgcagaaa	atccatgcat	6360
	catgagtgtc	tcagggccca	gaatatgaga	gcaggtagga	aacagagaca	tcttccatcc	6420
	ctgagaggca	gtgcggtcca	gtgggtgggg	acacgggctc	tgggtcaggt	ttgtgttgtt	6480
30	tgtttgtttg	ttttgagaca	gagtctcgct	ctattgccca	ggctggagtg	cagtgtcaca	6540
	atctcggctt	actgcaactt	ctgccttccc	ggattcaagt	gattctcctg	cctcagcctc	6600
	cagagtagct	gggattacag	gtgcgtgcca	ccacgcctgg	ctaatttttg	tatttttgat	6660
	agagacgggg	tttcaccatg	ttggccaggc	tagtctcgaa	ctcttgacct	caagtgatct	6720
	gcctgcctcg	gcctcccaaa	gtgctgggat	tacaggcgtg	agccaccaca	cccagcccca	6780
35	ggttggtgtt	tgaatctgag	gagactgaag	caccaagggg	ttaaatgttt	tgcccacagc	6840
	catacttggg	ctcagttcct	tgccctaccc	ctcacttgag	ctgcttagaa	cctggtgggc	6900

	acatgggcaa	taaccaggtc	acactgtttt	gtaccaagtg	ttatgggaat	ccaagatagg	6960
	agtaatttgc	tctgtggagg	ggatgaggga	tagtggttag	ggaaagcttc	acaaagtggg	7020
	tgttgcttag	agattttcca	ggtggagaag	ggggcttcta	ggcagaaggc	atageceaag	7080
	caaagactgc	aagtgcatgg	ctgctcatgg	gtagaagaga	atccaccatt	cctcaacatg	7140
5	taccgagtcc	ttgccatgtg	caaggcaaca	tgggggtacc	aggaattcca	agcaatgtcc	7200
	aaacctaggg	tctgctttct	gggacctgaa	gatacaggat	ggatcagccc	aggctgcaat	7260
	cccattacca	cgagggggaa	aaaaacctga	aggctaaatt	gtaggtcggg	ttagaggtta	7320
	tttatggaaa	gttatattct	acctacatgg	ggtctataag	cctggcgcca	atcagaaaag	7380
	gaacaaacaa	cagacctagc	tgggaggggc	agcattttgt	tgtagggggc	ggggcacatg	7440
10	ttctgggggt	acagccagac	tcagggcttg	tattaatagt	ctgagagtaa	gacagacaga	7500
	gggatagaag	gaaataggtc	cctttctctc	tctctctc	tctctctc	actctctctc	7560
	tctctcacac	acacacacag	acacacacac	acgctctgta	ggggtctact	tatgctccaa	7620
		ggccacattt					7680
		attccctgtt					7740
15		gaagccacct					7800
		ccacacccgc					7860
		ccagatcatt					7920
		ttcttggaac					7980
		gggcagcttt					8040
20		atattcctct					8100
		ctgtaggcca					8160
		gggcagtaaa					8220
		tcacacctgt					8280
		gttagagact					8340
25		gcaaggcatg					8400
		ggcttgaacc					8460
		ctggcaacag					8520
		gaacccaggt					8580
20		tcctgcagga					8640
30		tgctttagag					8700
		attcatcatt					8760
		tccctacaat					8820
		gagcagctgc					8880
2.5		gagagggtg					8940
35		agcagattta					9000
	aaggagaggg	tggggatgga	gaggaagaga	gggtgatcat	tcattcattc	cattgctact	9060

					gggcatgtgg		9120
	ggagcctcat	ggagctcaca	gggagtgctg	gcaaggagat	ggataatgga	cggataacaa	9180
	ataaacattt	agtacaatgt	ccgggaatgg	aaagttctcg	aaagaaaaat	aaagctggtg	9240
	agcatataga	cagccctgaa	ggcggccagg	ccaggcattt	ctgaggaggt	ggcatttgag	9300
5	С						9301
		0> 19					
		1> 21					
•		2> DNA					
10	<213	3> Artificia	al Sequence				
	.220	,					
	<220						
	<223	3> Primer fo	or PCR				
15	<400)> 19					
		, agaacaacaa	a				
	22 2 22	,	9				21
	<210	> 20					
	<211	> 19					
20	<212	> DNA					
	<213	> Artificia	1 Sequence				
	<220	>					
	<223	> PRimer fo	r PCR				
25							
	<400	> 20					
	gcactggccg	gagcacacc					19
••	<210:						
30	<211:						
		> DNA					
	<213:	> Artificia	l Sequence				
	200						
25	<220:						
35	<223:	> Primer for	r PCR				

	<400> 21	
	aggccaaccg cgagaagatg acc	23
	<210> 22	
5	<211> 21	
	<212> DNA	
	<213> Artificial Sequence	
	<220>	
10	<223> Primer for PCR	
	<400> 22	
	gaagtccagg gcgacgtagc a	21
15	<210> 23	
	<211> 25	
	<212> DNA	
	<213> Artificial Sequence	
20	<220>	
	<223> Primer for PCR	
	<400> 23	
	aagettggta ccatgeaget eecac	25
25		25
	<210> 24	
	<211> 50	
	<212> DNA	
30	<213> Artificial Sequence	
20	<220>	
	<223> Primer for PCR	
	4400. 24	
35	<400> 24	
35	aagcttctac ttgtcatcgt cgtccttgta gtcgtaggcg ttctccagct	50

```
<210> 25
              <211> 19
              <212> DNA
             <213> Artificial Sequence
   5
             <220>
             <223> Primer for PCR
             <400> 25
  10
       gcactggccg gagcacacc
                                                                                19
             <210> 26
             <211> 39
             <212> DNA
 15
             <213> Artificial Sequence
             <220>
             <223> Primer for PCR
 20
            <400> 26
      gtcgtcggat ccatggggtg gcaggcgttc aagaatgat
                                                                               39
            <210> 27
            <211> 57
25
            <212> DNA
            <213> Artificial Sequence
            <220>
            <223> Primer for PCR
30
            <400> 27
     gtcgtcaagc ttctacttgt catcgtcctt gtagtcgtag gcgttctcca gctcggc
                                                                              57
           <210> 28
35
           <211> 29
           <212> DNA
```

	<213> Artificial Sequence	
	<220>	
	<223> Primer for PCR	
5		
	<400> 28	
	gacttggatc ccaggggtgg caggcgttc	29
	<210> 29	
10	<211> 29	
	<212> DNA	·
	<213> Artificial Sequence	
	<220>	
15	<223> Primer for PCR	
	<400> 29	
	agcataagct totagtaggo gttotocag	
	-	29
20	<210> 30	
	<211> 29	
	<212> DNA	
	<213> Artificial Sequence	
25	<220>	
	<223> Primer for PCR	
	<400> 30	
	gacttggatc cgaagggaaa aagaaaggg	
30		29
	<210> 31	
	<211> 29	
	<212> DNA	
	<213> Artificial Sequence	
35		
	<220>	

	<223> Primer for PCR		
	<400> 31		
	agcataagct tttaatccaa atcgatgga		
5	January and account accounting		29
	<210> 32		
	<211> 33		
	<212> DNA		
	<213> Artificial Sequence		
10			
	<220>		
	<223> Primer for PCR		
	<400> 32		
15	actacgaget eggeeceace acceateace	aag	33
	<210> 33		
	<211> 34		
	<212> DNA		
20	<213> Artificial Sequence		
	<220>		
	<223> Primer for PCR		
25	<400> 33		
	acttagaagc tttcagtcct cagccccctc	ttcc	34
			24
	<210> 34		
	<211> 66		
30	<212> DNA		
	<213> Artificial Sequence		
	•		
	<220>		
	<223> Primer for PCR		
35			
	<400> 34		

	aatciggaic cataaciicg tatagcatac aitatacgaa gitaicigca ggaiicgagg	60
	gcccct	66
		00
	<210> 35	
5	<211> 82	
	<212> DNA	
	<213> Artificial Sequence	
	<220>	
10	<223> Primer for PCR	
	<400> 35	
	aatctgaatt ccaccggtgt taattaaata acttcgtata atgtatgcta tacgaagtta	60
	tagatotaga gtoagottot ga	82
15		02
	<210> 36	
	<211> 62	
	<212> DNA	
	<213> Artificial Sequence	
20		
	<220>	
	<223> Primer for PCR	
	<400> 36	
25	atttaggtga cactatagaa ctcgagcagc tgaagcttaa ccacatggtg gctcacaacc	60
	at	62
	<210> 37	
	<211> 54	
30	<212> DNA	
	<213> Artificial Sequence	
	<220>	
	<223> Primer for PCR	
35		
	<400> 37	

	aacgacggcc agtgaatccg taatcatggt catgctgcca ggtggaggag ggca	54
	<210> 38	
	<211> 31 <212> DNA	
5	<213> Artificial Sequence	
	<220>	
	<223> Primer for PCR	
10	<400> 38	
	attaccaccg gtgacacccg cttcctgaca g	31
		31
	<210> 39	
	<211> 61	
15	<212> DNA	
	<213> Artificial Sequence	
	• -	
	<220>	
20	<223> Primer for PCR	
20	4400. 30	
	<400> 39	
	attacttaat taaacatggc gcgccatatg gccggcccct aattgcggcg catcgttaat	60
		61
25	<210> 40	
	<211> 34	
	<212> DNA	
	<213> Artificial Sequence	
30	<220>	
	<223> Primer for PCR	
	 	
	<400> 40	
	attacggccg gccgcaaagg aattcaagat ctga	34
35		
	<210 > 41	

<211> 34

<212> DNA

<213> Artificial Sequence

5 <220>

<223> Primer for PCR

<400> 41

attacggcgc gcccctcaca ggccgcaccc agct

Intel Snal Application No PCT/US 99/27990

A. CLAS	SIFICATION OF SUBJEC	TMATTER		PCI	/US 99/27990	
1110 /	C12N15/12 C07K16/22 G01N33/53	C07K14/51 C12Q1/68 A01K67/027	C12N15/62	A61K38/18	C12N5/10 A61P19/10	
According	to International Patent Cla	ssification (IPC) or to both	national classification an	d IPC		
	S SEARCHED					
IPC 7	documentation searched (CO7K	ciassification system follo	wed by classification symb	pols)		
Document	ation searched other than	ninimum documentation to	o the extent that such doc	uments are included in	the fields searched	
Electronic	data base consulted during	the international search	(name of data base and,	where practical, search	terms used)	
					,	
C. DOCUM	ENTS CONSIDERED TO	RE REI EVANT				
Category 3		th indication, where appre				
					Relevant to claim No.	
X	EMBL SEQUEN	L.: "Homo sa RPC905N1, com CE DATABASE,	plete sequence	e"	1,2, 27-30	
	14 November 1997 (1997-11-14), XP002133385 HEIDELBERG DE Ac AC003098 the whole document					
X	HILLIER ET AL.: "WshU-Merck EST Project 1997" EMBL SEQUENCE DATABASE, 19 May 1997 (1997-05-19), XP002133386 HEIDELBERG DE Ac AA393939 the whole document				1,2, 27-30	
			-/			
	er documents are listed in t		X	Patent family members a	are listed in annex.	
Special cate	egories of cited documents	of the articles and	"T" later o	ocument published after	The international filter A	
E" earlier do filing dat L" document	cument but published on o te	r after the international	inver "X" docum cann	ntion nent of particular relevan of be considered povel	or cannot be considered to	
citation of citation of comments of the citation of citation of the citation of citation of ci	or other special reason (as it referring to an oral disclo- eans	cation date of another specified) sure, use, exhibition or	"Y" docum cann docu	nent of particular relevant to the considered to involve ment is combined with o	on the document is taken alone ice; the claimed invention we an inventive step when the	
	t published prior to the inte in the priority date claimed		in the	a, auch complination ball	ng obvious to a person skilled	
ate of the ac	aual complation of the inter	national search		of mailing of the internat		
	March 2000			07/04/2000		
ame and ma	iling address of the ISA European Patent Office, NL - 2280 HV Rijswijk	P.B. 5818 Patentiaan 2	Autho	rized officer		
	Tel. (+31-70) 340-2040 Fax: (+31-70) 340-3016	Tx. 31 651 epo n!,		Ceder, O		

Inte. onal Application No
PCT/US 99/27990

C.(Continu	ation) DOCUMENTS CONSIDERED TO BE RELEVANT	PCT/US 99/27990
Category 3	Citation of document, with indication, where appropriate, of the relevant passages	Relevant
		Relevant to claim No.
X	BONALDO ET AL.: "Normalization and substraction: two approches to faciliate gene discovery" EMBL SEQUENCE DATABASE, 4 September 1998 (1998-09-04), XP002133484 HEIDELBERG DE AC AII13131 the whole document & BONALDO ET AL.: "Normalization and substraction: two approches to faciliate gene discovery" GENOME RES, vol. 6, no. 9, 1996, pages 791-806,	1,27-30
	US 5 780 263 A (ADAMS MARK D ET AL) 14 July 1998 (1998-07-14) cited in the application column 1, line 11 - line 13 column 1, line 40 - line 42 column 1, line 66 -column 2, line 47 column 9, line 50 - line 53 column 11, line 15 - line 37	1-22,32, 61-67, 73-79
	US 5 453 492 A (BUETZOW RALF ET AL) 26 September 1995 (1995-09-26) abstract column 3, line 60 -column 8, line 30	1-3,8,9, 11-13, 15-22, 59,61-67
	WO 91 13152 A (LUDWIG INST CANCER RES) 5 September 1991 (1991-09-05) the whole document	1-3,8, 11,13, 15,17,32
	HSU D R ET AL: "The Xenopus dorsalizing factor Gremlin identifies a novel family of secretes proteins that antagonize BMP activities" MOLECULAR CELL,US,CELL PRESS, CAMBRIDGE, MA, vol. 1, no. 5, April 1998 (1998-04), pages 673-683, XP002113640 ISSN: 1097-2765 cited in the application abstract page 676, left-hand column, line 10 - line 14	17
	WO 92 06693 A (FOX CHASE CANCER CENTER) 30 April 1992 (1992-04-30)	

lisernational application No.

PCT/US 99/27990

Box I	Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)					
This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:						
1. X	Claims Nos.: 57 and 58 because they relate to subject matter not required to be searched by this Authority, namely: See PCT/ISA/210					
2.	Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:					
з	Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).					
Box II	Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)					
This Inte	mational Searching Authority found multiple inventions in this international application, as follows:					
1.	As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.					
2.	As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.					
3.	As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:					
4.	No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:					
Remark	on Protest The additional search fees were accompanied by the applicant's protest. No protest accompanied the payment of additional search fees.					

International Application No. PCT/US 99 27990

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 57 and 58 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.1

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

information on patent family members

Inte ional Application No PCT/US 99/27990

			101703 33727330		
Patent document cited in search report		Publication date	Patent family member(s)		Publication date
US 5780263	A 	14-07-1998	CA WO AU EP JP	2220912 A 9639486 A 2766595 A 0871705 A 11506918 T	12-12-1996 12-12-1996 24-12-1996 21-10-1996 22-06-1999
US 5453492	A	26-09-1995	NONE		
WO 9113152	A	05-09-1991	US AU CA DE DE EP JP	5177197 A 649026 B 7449591 A 2076979 A 69131572 D 69131572 T 0517779 A 5504888 T	05-01-1993 12-05-1994 18-09-1991 28-08-1991 07-10-1999 23-12-1999 16-12-1992 29-07-1993
WO 9206693	Α	30-04-1992	AU AU CA EP JP	662304 B 8957591 A 2094608 A 0554376 A 6502311 T	31-08-1995 20-05-1992 23-04-1992 11-08-1993 17-03-1994

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization International Bureau



(43) International Publication Date 8 June 2000 (08.06.2000)

PCT

(10) International Publication Number WO 00/32773 A1

- (51) International Patent Classification⁷: C12N 15/12, C07K 14/51, 14/495, C12N 15/63, 5/10, C07K 16/22, C12Q 1/68, C12N 15/62, A61K 38/18, A61P 19/10, G01N 33/53, A01K 67/027
- (21) International Application Number: PCT/US99/27990
- (22) International Filing Date:

24 November 1999 (24.11.1999)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

60/110,283

27 November 1998 (27.11.1998) L

- (71) Applicant (for all designated States except US1: DARWIN DISCOVERY LTD. [GB/GB]; Cambridge Science Park. Milton Road, Cambridge, Cambridgeshire CB4 4WE (GB).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): BRUNKOW, Mary, E. [US/US]: 9829 Triton Drive NW. Seattle. WA 98117 (US). GALAS, David, J. [US/US]: 854 Guanajuato Drive. Claremont. CA 91711 (US). KOVACEVICH, Brian [US/US]; 4308 N.E. 6th Place. Renton. WA 98059 (US). MULLIGAN, John, T. [US/US]; 5823 17th Avenue Northeast. Seattle. WA 98105 (US). PAEPER, Bryan, W. [US/US]; 1617 Summit Avenue #43. Seattle. WA 98122 (US). VAN NESS, Jeffrey [US/US]: 10020 49th Avenue Northeast. Seattle, WA 98125 (US). WINKLER, David, G. [US/US]: 7037 20th Avenue NE. Seattle. WA 98115 (US).

[Continued on next page]

(54) Title: COMPOSITIONS AND METHODS FOR INCREASING BONE MINERALIZATION

Common Cysteine Backbone

50 human-oremina pro NUMBAN-CEMBERUS PRO MILLLEGLEV LLPLGKTTRH QUORRONDESS SPYLLPRINGR ELPTGABLEA SRTAYTVGAL LLLLGTLLPA AEGKKKGSOG numan-cerderus.org - EEKPOLEVAV PIEVAT.SPA GEGGROREKH ESREGRENKK PEREMIESRO human-dan pro human-beer pro -HOLPLA LCLVCLLVHT 103 numan-oreal to.org AT, PPPOKAD HIDSEOTOSP COPGSRIRGE GGERGTAIRS FEYLESSOFA numan-ceroerus.pro SDSEPFPPGT OSLIOPIO.G MKMEKSPLRE EAKKFIGHEM FRKTPASOGY human-dan pro MERVENGAVE PAMELAAPPP AFRYVEGOGN DAFKBOATET TPELGEYPEP PPELENNXTH KRAENGGRPP human-green in pro LHYTERKYLK ROMCKTOPLK OTTHEEGONS RTTINGF CY GOOMSEY (FR numan-certerus.pro ILPIKSHEVH WETCRIVPES QIITHEGCEK VVVQNNL CF GKCGSVHFF numan-dan.pro INKLALEPOK SAKCEAKHIT QIVGHSGCEA KSIOKRA CL GOCESYSYPH numan-peer pro HMPFETKDVS EYSCREUNFT RYVTDGPCRS AKPYTELVCS GOCGPARLLP human-green in .pro HTRKEEGSFG SCSF CKP KKFTTHMYTL MCPSLOPPTK K KRVTRVSO .. GAADHSHT SCSH .. CLP AKFTTHALPL NCTELSSVIK V . VALVEE human-cerperus.pro hustan-dan.pro TEPOSTESLY HOSS ... CMP ADSMIETYTL ECPCHEEYPR YDKLYEKTLH human-deer.pro NATGRGWAR PSGPDFRCTP DEVRAGRYOL LCPGGEAPRA RKVPLVAS. ALCOHOLOGICA OF A RESIDENCE OF A RES numan-ceroerus pro COCKVKTEHE DGHILHAGSO DSFIPGVSAhusan-dan dro CSCOACGKEP SHEELSYNYD GEDGASSAG THAHPHHAH PGGGTPEPED husan-beer pro CKCKRI TREH HOSELKDEGT EAARPDIGAX PRPRARSAKA NDAELENAYhuman-greminn pro

(57) Abstract: A novel class or family of $TGF-\beta$ binding proteins is disclosed. Also disclosed are assays for selecting molecules for increasing bone mineralization and methods for utilizing such molecules.

human-cerberus pro

hucan-beer.pro

human-dan.pro PPGAPHTEEE SAED



- (74) Agent: MCMASTERS, David, D.: Seed and Berry LLP, Suite 6300, 701 Fifth Avenue. Seattle. WA 98104-7092 (US).
- (81) Designated States (national): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM). European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU,

MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

- with international search report
- (48) Date of publication of this corrected version:

13 December 2001

(15) Information about Correction: see PCT Gazette No. 50/2001 of 13 December 2001. Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/32773 PCT/US99/27990

COMPOSITIONS AND METHODS FOR INCREASING BONE MINERALIZATION

TECHNICAL FIELD

The present invention relates generally to pharmaceutical products and methods and, more specifically, to methods and compositions suitable for increasing the mineral content of bone. Such compositions and methods may be utilized to treat a wide variety of conditions, including for example, osteopenia, osteoporosis, fractures and other disorders in which low bone mineral density are a hallmark of the disease.

BACKGROUND OF THE INVENTION

Two or three distinct phases of changes to bone mass occur over the life of an individual (see Riggs, West J. Med. 154:63-77, 1991). The first phase occurs in both men and women, and proceeds to attainment of a peak bone mass. This first phase is achieved through linear growth of the endochondral growth plates, and radial growth due to a rate of periosteal apposition. The second phase begins around age 30 for trabecular bone (flat bones such as the vertebrae and pelvis) and about age 40 for cortical bone (e.g., long bones found in the limbs) and continues to old age. This phase is characterized by slow bone loss, and occurs in both men and women. In women, a third phase of bone loss also occurs, most likely due to postmenopausal estrogen deficiencies. During this phase alone, women may lose an additional 10% of bone mass from the cortical bone and 25% from the trabecular compartment (see Riggs, supra).

Loss of bone mineral content can be caused by a wide variety of conditions, and may result in significant medical problems. For example, osteoporosis is a debilitating disease in humans characterized by marked decreases in skeletal bone mass and mineral density, structural deterioration of bone including degradation of bone microarchitecture and corresponding increases in bone fragility and susceptibility to fracture in afflicted individuals. Osteoporosis in humans is preceded by clinical osteopenia (bone mineral density that is greater than one standard deviation but less than 2.5 standard deviations below the mean value for young adult bone), a condition found in approximately 25 million people in the United States. Another 7-8 million patients in the United States have been diagnosed with clinical osteoporosis (defined as bone mineral content greater than 2.5 standard deviations below that of mature young adult bone). Osteoporosis is one of the most expensive diseases for the health care

10

20

25

10

15

20

25

30

35

system, costing tens of billions of dollars annually in the United States. In addition to health care-related costs, long-term residential care and lost working days add to the financial and social costs of this disease. Worldwide approximately 75 million people are at risk for osteoporosis.

The frequency of osteoporosis in the human population increases with age, and among Caucasians is predominant in women (who comprise 80% of the osteoporosis patient pool in the United States). The increased fragility and susceptibility to fracture of skeletal bone in the aged is aggravated by the greater risk of accidental falls in this population. More than 1.5 million osteoporosis-related bone fractures are reported in the United States each year. Fractured hips, wrists, and vertebrae are among the most common injuries associated with osteoporosis. Hip fractures in particular are extremely uncomfortable and expensive for the patient, and for women correlate with high rates of mortality and morbidity.

Although osteoporosis has been defined as an increase in the risk of fracture due to decreased bone mass, none of the presently available treatments for skeletal disorders can substantially increase the bone density of adults. There is a strong perception among all physicians that drugs are needed which could increase bone density in adults, particularly in the bones of the wrist, spinal column and hip that are at risk in osteopenia and osteoporosis.

Current strategies for the prevention of osteoporosis may offer some benefit to individuals but cannot ensure resolution of the disease. These strategies include moderating physical activity (particularly in weight-bearing activities) with the onset of advanced age, including adequate calcium in the diet, and avoiding consumption of products containing alcohol or tobacco. For patients presenting with clinical osteopenia or osteoporosis, all current therapeutic drugs and strategies are directed to reducing further loss of bone mass by inhibiting the process of bone absorption, a natural component of the bone remodeling process that occurs constitutively.

For example, estrogen is now being prescribed to retard bone loss. There is, however, some controversy over whether there is any long term benefit to patients and whether there is any effect at all on patients over 75 years old. Moreover, use of estrogen is believed to increase the risk of breast and endometrial cancer.

High doses of dietary calcium, with or without vitamin D has also been suggested for postmenopausal women. However, high doses of calcium can often have unpleasant gastrointestinal side effects, and serum and urinary calcium levels must be continuously monitored (see Khosla and Rigss, Mayo Clin. Proc. 70:978-982, 1995).

20

25

30

35

Other therapeutics which have been suggested include calcitonin, bisphosphonates, anabolic steroids and sodium fluoride. Such therapeutics however, have undesirable side effects (e.g., calcitonin and steroids may cause nausea and provoke an immune reaction, bisphosphonates and sodium fluoride may inhibit repair of fractures, even though bone density increases modestly) that may prevent their usage (see Khosla and Rigss, supra).

No currently practiced therapeutic strategy involves a drug that stimulates or enhances the growth of new bone mass. The present invention provides compositions and methods which can be utilized to increase bone mineralization, and thus may be utilized to treat a wide variety of conditions where it is desired to increase bone mass. Further, the present invention provides other, related advantages.

SUMMARY OF THE INVENTION

As noted above, the present invention provides a novel class or family of TGF-beta binding-proteins, as well as assays for selecting compounds which increase bone mineral content and bone mineral density, compounds which increase bone mineral content and bone mineral density and methods for utilizing such compounds in the treatment or prevention of a wide variety of conditions.

Within one aspect of the present invention, isolated nucleic acid molecules are provided, wherein said nucleic acid molecules are selected from the group consisting of: (a) an isolated nucleic acid molecule comprising sequence ID Nos. 1, 5, 7, 9, 11, 13, or, 15, or complementary sequence thereof; (b) an isolated nucleic acid molecule that specifically hybridizes to the nucleic acid molecule of (a) under conditions of high stringency; and (c) an isolated nucleic acid that encodes a TGF-beta binding-protein according to (a) or (b). Within related aspects of the present invention, isolated nucleic acid molecules are provided based upon hybridization to only a portion of one of the above-identified sequences (e.g., for (a) hybridization may be to a probe of at least 20, 25, 50, or 100 nucleotides selected from nucleotides 156 to 539 or 555 to 687 of Sequence ID No. 1). As should be readily evident, the necessary stringency to be utilized for hybridization may vary based upon the size of the probe. For example, for a 25-mer probe high stringency conditions could include: 60 mM Tris pH 8.0, 2 mM EDTA, 5x Denhardt's, 6x SSC, 0.1% (w/v) N-laurylsarcosine, 0.5% (w/v) NP-40 (nonidet P-40) overnight at 45 degrees C, followed by two washes with with 0.2x SSC / 0.1% SDS at 45-50 degrees. For a 100-mer probe under low stringency conditions, suitable conditions might include the following: 5x SSPE, 5x Denhardt's, and 0.5% SDS overnight at 42-50 degrees, followed by two washes with 2x SSPE (or 2x SSC)

20

25

30

35

/0.1% SDS at 42-50 degrees.

Within related aspects of the present invention, isolated nucleic acid molecules are provided which have homology to Sequence ID Nos. 1, 5, 7, 9, 11, 13, or 15, at a 50%, 60%, 75%, 80%, 90%, 95%, or 98% level of homology utilizing a Wilbur-Lipman algorithm. Representative examples of such isolated molecules include, for example, nucleic acid molecules which encode a protein comprising Sequence ID NOs. 2, 6, 10, 12, 14, or 16, or have homology to these sequences at a level of 50%, 60%, 75%, 80%, 90%, 95%, or 98% level of homology utilizing a Lipman-Pearson algorithm.

Isolated nucleic acid molecules are typically less than 100kb in size, and, within certain embodiments, less than 50kb, 25kb, 10kb, or even 5kb in size. Further, isolated nucleic acid molecules, within other embodiments, do not exist in a "library" of other unrelated nucleic acid molecules (e.g., a subclone BAC such as described in GenBank Accession No. AC003098 and EMB No. AQ171546). However, isolated nucleic acid molecules can be found in libraries of related molecules (e.g., for shuffling, such as is described in U.S. Patent Nos. 5,837,458; 5,830,721; and 5,811,238). Finally, isolated nucleic acid molecules as described herein do not include nucleic acid molecules which encode Dan, Cerberus, Gremlin, or SCGF (U.S. Patent No. 5,780,263).

Also provided by the present invention are cloning vectors which contain the above-noted nucleic acid molecules, and expression vectors which comprise a promoter (e.g., a regulatory sequence) operably linked to one of the above-noted nucleic acid molecules. Representative examples of suitable promoters include tissue-specific promoters, and viral – based promoters (e.g., CMV-based promoters such as CMV I-E, SV40 early promoter, and MuLV LTR). Expression vectors may also be based upon, or derived from viruses (e.g., a "viral vector"). Representative examples of viral vectors include herpes simplex viral vectors, adenoviral vectors, adenovirus-associated viral vectors and retroviral vectors. Also provided are host cells containing or comprising any of above-noted vectors (including for example, host cells of human, monkey, dog, rat, or mouse origin).

Within other aspects of the present invention, methods of producing TGF-beta binding-proteins are provided, comprising the step of culturing the aforementioned host cell containing vector under conditions and for a time sufficient to produce the TGF-beta binding protein. Within further embodiments, the protein produced by this method may be further purified (e.g., by column chromatography, affinity purification, and the like). Hence, isolated proteins which are encoded by the

above-noted nucleic acid molecules (e.g., Sequence ID NOs. 2, 4, 6, 8, 10, 12, 14, or 16) may be readily produced given the disclosure of the subject application.

It should also be noted that the aforementioned proteins, or fragments thereof, may be produced as fusion proteins. For example, within one aspect fusion proteins are provided comprising a first polypeptide segment comprising a TGF-beta binding-protein encoded by a nucleic acid molecule as described above, or a portion thereof of at least 10, 20, 30, 50, or 100 amino acids in length, and a second polypeptide segment comprising a non-TGF-beta binding-protein. Within certain embodiments, the second polypeptide may be a tag suitable for purification or recognition (e.g., a polypeptide comprising multiple anionic amino acid residues – see U.S. Patent No. 4,851,341), a marker (e.g., green fluorescent protein, or alkaline phosphatase), or a toxic molecule (e.g., ricin).

Within another aspect of the present invention, antibodies are provided which are capable of specifically binding the above-described class of TGF-beta binding proteins (e.g., human BEER). Within various embodiments, the antibody may be a polyclonal antibody, or a monoclonal antibody (e.g., of human or murine origin). Within further embodiments, the antibody is a fragment of an antibody which retains the binding characteristics of a whole antibody (e.g., an F(ab')₂, F(ab)₂, Fab', Fab, or Fv fragment, or even a CDR). Also provided are hybridomas and other cells which are capable of producing or expressing the aforementioned antibodies.

Within related aspects of the invention, methods are provided detecting a TGF-beta binding protein, comprising the steps of incubating an antibody as described above under conditions and for a time sufficient to permit said antibody to bind to a TGF-beta binding protein, and detecting the binding. Within various embodiments the antibody may be bound to a solid support to facilitate washing or separation, and/or labeled. (e.g., with a marker selected from the group consisting of enzymes, fluorescent proteins, and radioisotopes).

Within other aspects of the present invention, isolated oligonucleotides are provided which hybridize to a nucleic acid molecule according to Sequence ID NOs. 1, 3, 5, 7, 9, 11, 13, 15, 17, or 18 or the complement thereto, under conditions of high stringency. Within further embodiments, the oligonucleotide may be found in the sequence which encodes Sequence ID Nos. 2, 4, 6, 8, 10, 12, 14, or 16. Within certain embodiments, the oligonucleotide is at least 15, 20, 30, 50, or 100 nucleotides in length. Within further embodiments, the oligonucleotide is labeled with another molecule (e.g., an enzyme, fluorescent molecule, or radioisotope). Also provided are primers which are capable of specifically amplifying all or a portion of the above-

15

20

25

30

10

15

20

25

30

35

mentioned nucleic acid molecules which encode TGF-beta binding-proteins. As utilized herein, the term "specifically amplifying" should be understood to refer to primers which amplify the aforementioned TGF-beta binding-proteins, and not other TGF-beta binding proteins such as Dan, Cerberus, Gremlin, or SCGF (U.S. Patent No. 5,780,263).

Within related aspects of the present invention, methods are provided for detecting a nucleic acid molecule which encodes a TGF-beta binding protein, comprising the steps of incubating an oligonucleotide as described above under conditions of high stringency, and detecting hybridization of said oligonucleotide. Within certain embodiments, the oligonucleotide may be labeled and/or bound to a solid support.

Within other aspects of the present invention, ribozymes are provided which are capable of cleaving RNA which encodes one of the above-mentioned TGF-beta binding-proteins (e.g., Sequence ID NOs. 2, 6, 8, 10, 12, 14, or 16). Such ribozymes may be composed of DNA, RNA (including 2'-O-methyl ribonucleic acids), nucleic acid analogs (e.g., nucleic acids having phosphorothioate linkages) or mixtures thereof. Also provided are nucleic acid molecules (e.g., DNA or cDNA) which encode these ribozymes, and vectors which are capable of expressing or producing the ribozymes. Representative examples of vectors include plasmids, retrotransposons, cosmids, and viral-based vectors (e.g., viral vectors generated at least in part from a retrovirus, adenovirus, or, adeno-associated virus). Also provided are host cells (e.g., human, dog, rat, or mouse cells) which contain these vectors. In certain embodiments, the host cell may be stably transformed with the vector.

Within further aspects of the invention, methods are provided for producing ribozymes either synthetically, or by in vitro or in vivo transcription. Within further embodiments, the ribozymes so produced may be further purified and/or formulated into pharmaceutical compositions (e.g., the ribozyme or nucleic acid molecule encoding the ribozyme along with a pharmaceutically acceptable carrier or diluent). Similarly, the antisense oligonucleotides and antibodies or other selected molecules described herein may be formulated into pharmaceutical compositions.

Within other aspects of the present invention, antisense oligonucleotides are provided comprising a nucleic acid molecule which hybridizes to a nucleic acid molecule according to Sequence ID NOs. 1, 3, 5, 7, 9, 11, 13, or 15, or the complement thereto, and wherein said oligonucleotide inhibits the expression of TGF-beta binding protein as described herein (e.g., human BEER). Within various embodiments, the oligonucleotide is 15, 20, 25, 30, 35, 40, or 50 nucleotides in length. Preferably, the

20

25

30

oligonucleotide is less than 100, 75, or 60 nucleotides in length. As should be readily evident, the oligonucleotide may be comprised of one or more nucleic acid analogs, ribonucleic acids, or deoxyribonucleic acids. Further, the oligonucleotide may be modified by one or more linkages, including for example, covalent linkage such as a phosphorothioate linkage, a phosphotriester linkage, a methyl phosphonate linkage, a methylene(methylimino) linkage, a morpholino linkage, an amide linkage, a polyamide linkage, a short chain alkyl intersugar linkage, a cycloalkyl intersugar linkage, a short chain heteroatomic intersugar linkage and a heterocyclic intersugar linkage. One representative example of a chimeric oligonucleotide is provied in U.S. Patent No. 5,989,912.

Within yet another aspect of the present invention, methods are provided for increasing bone mineralization, comprising introducing into a warm-blooded animal an effective amount of the ribozyme as described above. Within related aspects, such methods comprise the step of introducing into a patient an effective amount of the nucleic acid molecule or vector as described herein which is capable of producing the desired ribozyme, under conditions favoring transcription of the nucleic acid molecule to produce the ribozyme.

Within other aspects of the invention transgenic, non-human animals are provided. Within one embodiment a transgenic animal is provided whose germ cells and somatic cells contain a nucleic acid molecule encoding a TGF-beta binding-protein as described above which is operably linked to a promoter effective for the expression of the gene, the gene being introduced into the animal, or an ancestor of the animal, at an embryonic stage, with the proviso that said animal is not a human. Within other embodiments, transgenic knockout animals are provided, comprising an animal whose germ cells and somatic cells comprise a disruption of at least one allele of an endogenous nucleic acid molecule which hybridizes to a nucleic acid molecule which encodes a TGF-binding protein as described herein, wherein the disruption prevents transcription of messenger RNA from said allele as compared to an animal without the disruption, with the proviso that the animal is not a human. Within various embodiments, the disruption is a nucleic acid deletion, substitution, or, insertion. Within other embodiments the transgenic animal is a mouse, rat, sheep, pig, or dog.

Within further aspects of the invention, kits are provided for the detection of TGF-beta binding-protein gene expression, comprising a container that comprises a nucleic acid molecule, wherein the nucleic acid molecule is selected from the group consisting of (a) a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, or 15; (b) a nucleic acid molecule comprising the

15

20

30

35

complement of the nucleotide sequence of (a); (c) a nucleic acid molecule that is a fragment of (a) or (b) of at least 15, 20 30, 50, 75, or, 100 nucleotides in length. Also provided are kits for the detection of a TGF-beta binding-protein which comprise a container that comprise one of the TGF-beta binding protein antibodies described herein.

For example, within one aspect of the present invention methods are provided for determining whether a selected molecule is capable of increasing bone mineral content, comprising the steps of (a) mixing one or more candidate molecules with TGF-beta-binding-protein encoded by the nucleic acid molecule according to claim 1 and a selected member of the TGF-beta family of proteins (e.g., BMP 5 or 6), (b) determining whether the candidate molecule alters the signaling of the TGF-beta family member, or alters the binding of the TGF-beta binding-protein to the TGF-beta family member. Within certain embodiments, the molecule alters the ability of TGF-beta to function as a positive regulator of mesenchymal cell differentiation. Within this aspect of the present invention, the candidate molecule(s) may alter signaling or binding by, for example, either decreasing (e.g., inhibiting), or increasing (e.g., enhancing) signaling or binding.

Within yet another aspect, methods are provided for determining whether a selected molecule is capable of increasing bone mineral content, comprising the step of determining whether a selected molecule inhibits the binding of TGF-beta binding-protein to bone, or an analogue thereof. Representative examples of bone or analogues thereof include hydroxyapatite and primary human bone samples obtained via biopsy.

Within certain embodiments of the above-recited methods, the selected molecule is contained within a mixture of molecules and the methods may further comprise the step of isolating one or more molecules which are functional within the assay. Within yet other embodiments, TGF-beta family of proteins is bound to a solid support and the binding of TGF-beta binding-protein is measured or TGF-beta binding-protein are bound to a solid support and the binding of TGF-beta proteins are measured.

Utilizing methods such as those described above, a wide variety of molecules may be assayed for their ability to increase bone mineral content by inhibiting the binding of the TGF-beta binding-protein to the TGF-beta family of proteins. Representative examples of such molecules include proteins or peptides, organic molecules, and nucleic acid molecules.

Within other related aspects of the invention, methods are provided for increasing bone mineral content in a warm-blooded animal, comprising the step of

15

20

25

30

35

administering to a warm-blooded animal a therapeutically effective amount of a molecule identified from the assays recited herein. Within another aspect, methods are provided for increasing bone mineral content in a warm-blooded animal, comprising the step of administering to a warm-blooded animal a therapeutically effective amount of a molecule which inhibits the binding of the TGF-beta binding-protein to the TGF-beta super-family of proteins, including bone morphogenic proteins (BMPs). Representative examples of suitable molecules include antisense molecules, ribozymes, ribozyme genes, and antibodies (e.g., a humanized antibody) which specifically recognize and alter the activity of the TGF-beta binding-protein.

Within another aspect of the present invention, methods are provided for increasing bone mineral content in a warm-blooded animal, comprising the steps of (a) introducing into cells which home to the bone a vector which directs the expression of a molecule which inhibits the binding of the TGF-beta binding-protein to the TGF-beta family of proteins and bone morphogenic proteins (BMPs), and (b) administering the vector-containing cells to a warm-blooded animal. As utilized herein, it should be understood that cells "home to bone" if they localize within the bone matrix after peripheral administration. Within one embodiment, such methods further comprise, prior to the step of introducing, isolating cells from the marrow of bone which home to the bone. Within a further embodiment, the cells which home to bone are selected from the group consisting of CD34+ cells and osteoblasts.

Within other aspects of the present invention, molecules are provided (preferably isolated) which inhibit the binding of the TGF-beta binding-protein to the TGF-beta super-family of proteins.

Within further embodiments, the molecules may be provided as a composition, and can further comprise an inhibitor of bone resorption. Representative examples of such inhibitors include calcitonin, estrogen, a bisphosphonate, a growth factor having anti-resorptive activity and tamoxifen.

Representative examples of molecules which may be utilized in the afore-mentioned therapeutic contexts include, e.g., ribozymes, ribozyme genes, antisense molecules, and/or antibodies (e.g., humanized antibodies). Such molecules may depending upon their selection, used to alter, antagonize, or agonize the signalling or binding of a TGF-beta binding-protein family member as described herein

Within various embodiments of the invention, the above-described molecules and methods of treatment or prevention may be utilized on conditions such as osteoporosis, osteomalasia, periodontal disease, scurvy, Cushing's Disease, bone fracture and conditions due to limb immobilization and steroid usage.

Ю

15

20

25

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth herein which describe in more detail certain procedures or compositions (e.g., plasmids, etc.), and are therefore incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic illustration comparing the amino acid sequence of Human Dan; Human Gremlin; Human Cerberus and Human Beer. Arrows indicate the Cysteine backbone.

Figure 2 summarizes the results obtained from surveying a variety of human tissues for the expression of a TGF-beta binding-protein gene, specifically, the Human Beer gene. A semi-quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) procedure was used to amplify a portion of the gene from first-strand cDNA synthesized from total RNA (described in more detail in EXAMPLE 2A).

Figure 3 summarizes the results obtained from RNA in situ hybridization of mouse embryo sections, using a cRNA probe that is complementary to the mouse Beer transcript (described in more detail in EXAMPLE 2B). Panel A is a transverse section of 10.5 dpc embryo. Panel B is a sagittal section of 12.5 dpc embryo and panels C and D are sagittal sections of 15.5 dpc embryos.

Figure 4 illustrates, by western blot analysis, the specificity of three different polyclonal antibodies for their respective antigens (described in more detail in EXAMPLE 4). Figure 4A shows specific reactivity of an anti-H. Beer antibody for H. Beer antigen, but not H. Dan or H. Gremlin. Figure 4B shows reactivity of an anti-H. Gremlin antibody for H. Gremlin antigen, but not H. Beer or H. Dan. Figure 4C shows reactivity of an anti-H. Dan antibody for H. Dan, but not H. Beer or H. Gremlin.

Figure 5 illustrates, by western blot analysis, the selectivity of the TGF-beta binding-protein, Beer, for BMP-5 and BMP-6, but not BMP-4 (described in more detail in EXAMPLE 5).

Figure 6 demonstrates that the ionic interaction between the TGF-beta binding-protein, Beer, and BMP-5 has a dissociation constant in the 15-30 nM range.

WO 00/32773

10

15

20

25

30

35

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

Prior to setting forth the invention in detail, it may be helpful to an understanding thereof to set forth definitions of certain terms and to list and to define the abbreviations that will be used hereinafter.

"Molecule" should be understood to include proteins or peptides (e.g., antibodies, recombinant binding partners, peptides with a desired binding affinity), nucleic acids (e.g., DNA, RNA, chimeric nucleic acid molecules, and nucleic acid analogues such as PNA); and organic or inorganic compounds.

"TGF-beta" should be understood to include any known or novel member of the TGF-beta super-family, which also includes bone morphogenic proteins (BMPs).

"TGF-beta receptor" should be understood to refer to the receptor specific for a particular member of the TGF-beta super-family (including bone morphogenic proteins (BMPs)).

"TGF-beta binding-protein" should be understood to refer to a protein with specific binding affinity for a particular member or subset of members of the TGF-beta super-family (including bone morphogenic proteins (BMPs)). Specific examples of TGF-beta binding-proteins include proteins encoded by Sequence ID Nos. 1, 5, 7, 9, 11, 13, and 15.

Inhibiting the "binding of the TGF-beta binding-protein to the TGF-beta family of proteins and bone morphogenic proteins (BMPs)" should be understood to refer to molecules which allow the activation of TGF-beta or bone morphogenic proteins (BMPs), or allow the binding of TGF-beta family members including bone morphogenic proteins (BMPs) to their respective receptors, by removing or preventing TGF-beta from binding to TGF-binding-protein. Such inhibition may be accomplished, for example, by molecules which inhibit the binding of the TGF-beta binding-protein to specific members of the TGF-beta super-family.

"Vector" refers to an assembly which is capable of directing the expression of desired protein. The vector must include transcriptional promoter elements which are operably linked to the gene(s) of interest. The vector may be composed of either deoxyribonucleic acids ("DNA"), ribonucleic acids ("RNA"), or a combination of the two (e.g., a DNA-RNA chimeric). Optionally, the vector may include a polyadenylation sequence, one or more restriction sites, as well as one or more selectable markers such as neomycin phosphotransferase or hygromycin phosphotransferase. Additionally, depending on the host cell chosen and the vector

WO 00/32773

10

15

20

25

30

employed, other genetic elements such as an origin of replication, additional nucleic acid restriction sites, enhancers, sequences conferring inducibility of transcription, and selectable markers, may also be incorporated into the vectors described herein.

An "isolated nucleic acid molecule" is a nucleic acid molecule that is not integrated in the genomic DNA of an organism. For example, a DNA molecule that encodes a TGF-binding protein that has been separated from the genomic DNA of a eukaryotic cell is an isolated DNA molecule. Another example of an isolated nucleic acid molecule is a chemically-synthesized nucleic acid molecule that is not integrated in the genome of an organism. The isolated nucleic acid molecule may be genomic DNA, cDNA, RNA, or composed at least in part of nucleic acid analogs.

An "isolated polypeptide" is a polypeptide that is essentially free from contaminating cellular components, such as carbohydrate, lipid, or other proteinaceous impurities associated with the polypeptide in nature. Within certain embodiments, a particular protein preparation contains an isolated polypeptide if it appears nominally as a single band on SDS-PAGE gel with Coomassie Blue staining. "Isolated" when referring to organic molecules means that the compounds are greater than 90 percent pure utilizing methods which are well known in the art (e.g., NMR, melting point).

"Sclerosteosis" Sclerosteosis is a term that was applied by Hansen (1967) (Hansen, H. G., Sklerosteose.In: Opitz, H., Schmid, F., Handbuch der Kinderheilkunde. Berlin: Springer (pub.) 6 1967. Pp. 351-355) to a disorder similar to van Buchem hyperostosis corticalis generalisata but possibly differing in radiologic appearance of the bone changes and in the presence of asymmetric cutaneous syndactyly of the index and middle fingers in many cases. The jaw has an unusually square appearance in this condition.

"<u>Humanized antibodies</u>" are recombinant proteins in which murine complementary determining regions of monoclonal antibodies have been transferred from heavy and light variable chains of the murine immunoglobulin into a human variable domain.

As used herein, an "antibody fragment" is a portion of an antibody such as F(ab')₂, F(ab)₂, Fab', Fab, and the like Regardless of structure, an antibody fragment binds with the same antigen that is recognized by the intact antibody. For example, an anti-TGF-beta binding-protein monoclonal antibody fragment binds with an epitope of TGF-beta binding-protein.

The term "antibody fragment" also includes any synthetic or genetically engineered protein that acts like an antibody by binding to a specific antigen to form a complex. For example, antibody fragments include isolated fragments consisting of the

light chain variable region, "Fv" fragments consisting of the variable regions of the heavy and light chains, recombinant single chain polypeptide molecules in which light and heavy variable regions are connected by a peptide linker ("sFv proteins"), and minimal recognition units consisting of the amino acid residues that mimic the hypervariable region.

A "detectable label" is a molecule or atom which can be conjugated to an antibody moiety to produce a molecule useful for diagnosis. Examples of detectable labels include chelators, photoactive agents, radioisotopes, fluorescent agents, paramagnetic ions, enzymes, and other marker moieties.

As used herein, an "<u>immunoconjugate</u>" is a molecule comprising an anti-TGF-beta binding-protein antibody, or an antibody fragment, and a detectable label. An immunoconjugate has roughly the same, or only slightly reduced, ability to bind TGF-beta binding-protein after conjugation as before conjugation.

Abbreviations: TGF-beta – "Transforming Growth Factor-beta"; TGF-bBP – "Transforming Growth Factor-beta binding-protein" (one representative TGF-bBP is designated "H. Beer"); BMP – "bone morphogenic protein"; PCR – "polymerase chain reaction"; RT-PCR - PCR process in which RNA is first transcribed into DNA at the first step using reverse transcriptase (RT); cDNA - any DNA made by copying an RNA sequence into DNA form.

20

25

30

10

As noted above, the present invention provides a novel class of TGF-beta binding-proteins, as well as methods and compositions for increasing bone mineral content in warm-blooded animals. Briefly, the present inventions are based upon the unexpected discovery that a mutation in the gene which encodes a novel member of the TGF-beta binding-protein family results in a rare condition (sclerosteosis) characterized by bone mineral contents which are one- to four-fold higher than in normal individuals. Thus, as discussed in more detail below this discovery has led to the development of assays which may be utilized to select molecules which inhibit the binding of the TGF-beta binding-protein to the TGF-beta family of proteins and bone morphogenic proteins (BMPs), and methods of utilizing such molecules for increasing the bone mineral content of warm-blooded animals (including for example, humans).

DISCUSSION OF THE DISEASE KNOWN AS SCLEROSTEOSIS

Sclerosteosis is a term that was applied by Hansen (1967) (Hansen, H. G., Sklerosteose, In: Opitz, H.; Schmid, F., Handbuch der Kinderheilkunde. Berlin: Springer (pub.) 6 1967. Pp. 351-355) to a disorder similar to van Buchem hyperostosis

15

20

25

30

35

corticalis generalisata but possibly differing in radiologic appearance of the bone changes and in the presence of asymmetric cutaneous syndactyly of the index and middle fingers in many cases.

Sclerosteosis is now known to be an autosomal semi-dominant disorder which is characterized by widely disseminated sclerotic lesions of the bone in the adult. The condition is progressive. Sclerosteosis also has a developmental aspect which is associated with syndactyly (two or more fingers are fused together). The Sclerosteosis Syndrome is associated with large stature and many affected individuals attain a height of six feet or more. The bone mineral content of homozygotes can be 1 to 6 fold over normal individuals and bone mineral density can be 1 to 4 fold above normal values (e.g., from unaffected siblings).

The Sclerosteosis Syndrome occurs primarily in Afrikaaners of Dutch descent in South Africa. Approximately 1/140 individuals in the Afrikaaner population are carriers of the mutated gene (heterozygotes). The mutation shows 100% penetrance. There are anecdotal reports of increased of bone mineral density in heterozygotes with no associated pathologies (syndactyly or skull overgrowth).

It appears at the present time that there is no abnormality of the pituitary-hypothalamus axis in Sclerosteosis. In particular, there appears to be no over-production of growth hormone and cortisone. In addition, sex hormone levels are normal in affected individuals. However, bone turnover markers (osteoblast specific alkaline phosphatase, osteocalcin, type 1 procollagen C' propeptide (PICP), and total alkaline phosphatase; (see Comier, C., Curr. Opin. in Rheu. 7:243, 1995) indicate that there is hyperosteoblastic activity associated with the disease but that there is normal to slightly decreased osteoclast activity as measured by markers of bone resorption (pyridinoline, deoxypryridinoline, N-telopeptide, urinary hydroxyproline, plasma tartrate-resistant acid phosphatases and galactosyl hydroxylysine (see Comier, supra)).

Sclerosteosis is characterized by the continual deposition of bone throughout the skeleton during the lifetime of the affected individuals. In homozygotes the continual deposition of bone mineral leads to an overgrowth of bone in areas of the skeleton where there is an absence of mechanoreceptors (skull, jaw, cranium). In homozygotes with Sclerosteosis, the overgrowth of the bones of the skull leads to cranial compression and eventually to death due to excessive hydrostatic pressure on the brain stem. In all other parts of the skeleton there is a generalized and diffuse sclerosis. Cortical areas of the long bones are greatly thickened resulting in a substantial increase in bone strength. Trabecular connections are increased in thickness

which in turn increases the strength of the trabecular bone. Sclerotic bones appear unusually opaque to x-rays.

As described in more detail in Example 1, the rare genetic mutation that is responsible for the Sclerosteosis syndrome has been localized to the region of human chromosome 17 that encodes a novel member of the TGF-beta binding-protein family (one representative example of which is designated "H. Beer"). As described in more detail below, based upon this discovery, the mechanism of bone mineralization is more fully understood, allowing the development of assays for molecules which increase bone mineralization, and use of such molecules to increase bone mineral content, and in the treatment or prevention of a wide number of diseases.

TGF-BETA SUPER-FAMILY

The Transforming Growth Factor-beta (TGF-beta) super-family contains a variety of growth factors that share common sequence elements and structural motifs (at both the secondary and tertiary levels). This protein family is known to exert a wide spectrum of biological responses on a large variety of cell types. Many of them have 15 important functions during the embryonal development in pattern formation and tissue specification; in adults they are involved, e.g., in wound healing and bone repair and bone remodeling, and in the modulation of the immune system. In addition to the three TGF-beta's, the super-family includes the Bone Morphogenic Proteins (BMPs), Activins, Inhibins, Growth and Differentiation Factors (GDFs), and Glial-Derived 20 Neurotrophic Factors (GDNFs). Primary classification is established through general sequence features that bin a specific protein into a general sub-family. Additional stratification within the sub-family is possible due to stricter sequence conservation between members of the smaller group. In certain instances, such as with BMP-5, BMP-6 and BMP-7, this can be as high as 75 percent amino acid homology between members of the smaller group. This level of identity enables a single representative sequence to illustrate the key biochemical elements of the sub-group that separates it from other members of the larger family.

TGF-beta signals by inducing the formation of hetero-oligomeric complexes of type I and type II receptors. The crystal structure of TGF-beta2 has been determined. The general fold of the TGF-beta2 monomer contains a stable, compact, cysteine knotlike structure formed by three disulphide bridges. Dimerization, stabilized by one disulphide bridge, is antiparallel.

TGF-beta family members initiate their cellular action by binding to receptors with intrinsic serine/threonine kinase activity. This receptor family consists

30

of two subfamilies, denoted type I and type II receptors. Each member of the TGF-beta family binds to a characteristic combination of type I and type II receptors, both of which are needed for signaling. In the current model for TGF-beta receptor activation, TGF-beta first binds to the type II receptor (TbR-II), which occurs in the cell membrane in an oligomeric form with activated kinase. Thereafter, the type I receptor (TbR-I), which can not bind ligand in the absence of TbR-II, is recruited into the complex. TbR-II then phosphorylates TbR-I predominantly in a domain rich in glycine and serine residues (GS domain) in the juxtamembrane region, and thereby activates TbR-I.

Thus far seven type I receptors and five type II receptors have been identified.

BONE MORPHOGENIC PROTEINS (BMPs) ARE KEY REGULATORY PROTEINS IN DETERMINING BONE MINERAL DENSITY IN HUMANS

A major advance in the understanding of bone formation was identification of the bone morphogenic proteins (BMPs), also known as osteogenic proteins (OPs), which regulate cartilage and bone differentiation in vivo. BMPs/OPs induce endochondral bone differentiation through a cascade of events which include formation of cartilage, hypertrophy and calcification of the cartilage, vascular invasion, differentiation of osteoblasts, and formation of bone. As described above, the BMPs/OPs (BMP 2-14, and osteogenic protein 1 and -2, OP-1 and OP-2) are members of the TGF-beta super-family. The striking evolutionary conservation between members the BMP/OP sub-family suggests that they are critical in the normal development and function of animals. Moreover, the presence of multiple forms of BMPs/OPs raises an important question about the biological relevance of this apparent redundancy. In addition to postfetal chondrogenesis and osteogenesis, the BMPs/OPs play multiple roles in skeletogenesis (including the development of craniofacial and dental tissues) and in embryonic development and organogenesis of parenchymatous organs, including the kidney. It is now understood that nature relies on common (and few) molecular mechanisms tailored to provide the emergence of specialized tissues and organs. The BMP/OP super-family is an elegant example of nature parsimony in programming multiple specialized functions deploying molecular isoforms with minor variation in amino acid motifs within highly conserved carboxy-terminal regions.

BMP ANTAGONISM

The BMP and Activin sub-families are subject to significant post-

15

20

translational regulation. An intricate extracellular control system exists, whereby a high affinity antagonist is synthesized and exported, and subsequently complexes selectively with BMPs or activins to disrupt their biological activity (W.C. Smith (1999) TIG 15(1) 3-6). A number of these natural antagonists have been identified, and based on sequence divergence appear to have evolved independently due to the lack of primary sequence conservation. There has been no structural work to date on this class of proteins. Studies of these antagonists has highlighted a distinct preference for interacting and neutralizing BMP-2 and BMP-4. Furthermore, the mechanism of inhibition seems to differ for the different antagonists (S. lemura et al. (1998) Proc Natl Acad Sci USA 95 9337-9342).

NOVEL TGF-BETA BINDING-PROTEINS

1. Background re: TGF-beta binding-proteins

As noted above, the present invention provides a novel class of TGF-beta binding-proteins that possess a nearly identical cysteine (disulfide) scaffold when compared to Human DAN, Human Gremlin, and Human Cerberus, and SCGF (U.S. Patent No. 5,780,263) but almost no homology at the nucleotide level (for background information, see generally Hsu, D.R., Economides, A.N., Wang, X., Eimon, P.M., Harland, R.M., "The *Xenopus* Dorsalizing Factor Gremlin Identifies a Novel Family of Secreted Proteins that Antagonize BMP Activities," *Molecular Cell* 1:673-683, 1998).

One representative example of the novel class of TGF-beta binding-proteins is disclosed in Sequence ID Nos. 1, 5, 9, 11, 13, and 15. Representative members of this class of binding proteins should also be understood to include variants of the TGF-beta binding-protein (e.g., Sequence ID Nos. 5 and 7). As utilized herein, a "TGF-beta binding-protein variant gene" refers to nucleic acid molecules that encode a polypeptide having an amino acid sequence that is a modification of SEQ ID Nos. 2, 10, 12, 14 or 16. Such variants include naturally-occurring polymorphisms or allelic variants of TGF-beta binding-protein genes, as well as synthetic genes that contain conservative amino acid substitutions of these amino acid sequences. Additional variant forms of a TGF-beta binding-protein gene are nucleic acid molecules that contain insertions or deletions of the nucleotide sequences described herein. TGF-beta binding-protein variant genes can be identified by determining whether the genes hybridize with a nucleic acid molecule having the nucleotide sequence of SEQ ID Nos: 1, 5, 7, 9, 11, 13, or 15 under stringent conditions. In addition, TGF-beta binding-protein variant genes should encode a protein having a cysteine backbone.

15

20

25

As an alternative, TGF-beta binding-protein variant genes can be identified by sequence comparison. As used herein, two amino acid sequences have "100% amino acid sequence identity" if the amino acid residues of the two amino acid sequences are the same when aligned for maximal correspondence. Similarly, two nucleotide sequences have "100% nucleotide sequence identity" if the nucleotide residues of the two nucleotide sequences are the same when aligned for maximal correspondence. Sequence comparisons can be performed using standard software programs such as those included in the LASERGENE bioinformatics computing suite, which is produced by DNASTAR (Madison, Wisconsin). Other methods for comparing two nucleotide or amino acid sequences by determining optimal alignment are well-known to those of skill in the art (see, for example, Peruski and Peruski, The Internet and the New Biology: Tools for Genomic and Molecular Research (ASM Press, Inc. 1997), Wu et al. (eds.), "Information Superhighway and Computer Databases of Nucleic Acids and Proteins," in Methods in Gene Biotechnology, pages 123-151 (CRC Press, Inc. 1997), and Bishop (ed.), Guide to Human Genome Computing, 2nd Edition (Academic Press, Inc. 1998)).

A variant TGF-beta binding-protein should have at least a 50% amino acid sequence identity to SEQ ID NOs: 2, 6, 10, 12, 14 or 16 and preferably, greater than 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% identity. Alternatively, TGF-beta binding-protein variants can be identified by having at least a 70% nucleotide sequence identity to SEQ ID NOs: 1, 5, 9, 11, 13 or 15. Moreover, the present invention contemplates TGF-beta binding-protein gene variants having greater than 75%, 80%, 85%, 90%, or 95% identity to SEQ ID NO:1. Regardless of the particular method used to identify a TGF-beta binding-protein variant gene or variant TGF-beta bindingprotein, a variant TGF-beta binding-protein or a polypeptide encoded by a variant TGF-beta binding-protein gene can be functionally characterized by, for example, its ability to bind to and/or inhibit the signaling of a selected member of the TGF-beta family of proteins, or by its ability to bind specifically to an anti-TGF-beta bindingprotein antibody.

30 The present invention includes functional fragments of TGF-beta binding-protein genes. Within the context of this invention, a "functional fragment" of a TGF-beta binding-protein gene refers to a nucleic acid molecule that encodes a portion of a TGF-beta binding-protein polypeptide which either (1) possesses the above-noted function activity, or (2) specifically binds with an anti-TGF-beta bindingprotein antibody. For example, a functional fragment of a TGF-beta binding-protein gene described herein comprises a portion of the nucleotide sequence of SEQ ID Nos:

1, 5, 9, 11, 13, or 15.

2. Isolation of the TGF-beta binding-protein gene

DNA molecules encoding a binding-protein gene can be obtained by screening a human cDNA or genomic library using polynucleotide probes based upon, for example, SEQ ID NO:1.

For example, the first step in the preparation of a cDNA library is to isolate RNA using methods well-known to those of skill in the art. In general, RNA isolation techniques must provide a method for breaking cells, a means of inhibiting RNase-directed degradation of RNA, and a method of separating RNA from DNA, protein, and polysaccharide contaminants. For example, total RNA can be isolated by freezing tissue in liquid nitrogen, grinding the frozen tissue with a mortar and pestle to lyse the cells, extracting the ground tissue with a solution of phenol/chloroform to remove proteins, and separating RNA from the remaining impurities by selective precipitation with lithium chloride (see, for example, Ausubel et al. (eds.), Short Protocols in Molecular Biology, 3rd Edition, pages 4-1 to 4-6 (John Wiley & Sons 1995) ["Ausubel (1995)"]; Wu et al., Methods in Gene Biotechnology, pages 33-41 (CRC Press, Inc. 1997) ["Wu (1997)"]).

Alternatively, total RNA can be isolated by extracting ground tissue with guanidinium isothiocyanate, extracting with organic solvents, and separating RNA from contaminants using differential centrifugation (see, for example, Ausubel (1995) at pages 4-1 to 4-6; Wu (1997) at pages 33-41).

In order to construct a cDNA library, poly(A) RNA must be isolated from a total RNA preparation. Poly(A) RNA can be isolated from total RNA by using the standard technique of oligo(dT)-cellulose chromatography (see, for example, Ausubel (1995) at pages 4-11 to 4-12).

Double-stranded cDNA molecules are synthesized from poly(A) RNA using techniques well-known to those in the art. (see, for example, Wu (1997) at pages 41-46). Moreover, commercially available kits can be used to synthesize double-stranded cDNA molecules. For example, such kits are available from Life Technologies, Inc. (Gaithersburg, Maryland), CLONTECH Laboratories, Inc. (Palo Alto, California), Promega Corporation (Madison, Wisconsin) and Stratagene Cloning Systems (La Jolla, California).

The basic approach for obtaining TGF-beta binding-protein cDNA clones can be modified by constructing a subtracted cDNA library which is enriched in TGF-binding-protein-specific cDNA molecules. Techniques for constructing subtracted libraries are well-known to those of skill in the art (see, for example, Sargent, "Isolation of

20

25

30

Differentially Expressed Genes," in *Meth. Enzymol. 152*:423, 1987, and Wu et al. (eds.), "Construction and Screening of Subtracted and Complete Expression cDNA Libraries," in *Methods in Gene Biotechnology*, pages 29-65 (CRC Press, Inc. 1997)).

Various cloning vectors are appropriate for the construction of a cDNA library. For example, a cDNA library can be prepared in a vector derived from bacteriophage, such as a \(\lambda\gamma\text{t10}\) vector (see, for example, Huynh et al., "Constructing and Screening cDNA Libraries in \(\lambda\gamma\text{t10}\) and \(\lambda\gamma\text{t11}\)," in \(DNA\) (loning: A Practical Approach Vol. I, Glover (ed.), page 49 (IRL Press, 1985); Wu (1997) at pages 47-52).

Alternatively, double-stranded cDNA molecules can be inserted into a plasmid vector, such as a pBluescript vector (Stratagene Cloning Systems; La Jolla, California), a LambdaGEM-4 (Promega Corp.; Madison, Wisconsin) or other commercially available vectors. Suitable cloning vectors also can be obtained from the American Type Culture Collection (Rockville, Maryland).

In order to amplify the cloned cDNA molecules, the cDNA library is inserted into a prokaryotic host, using standard techniques. For example, a cDNA library can be introduced into competent *E. coli* DH5 cells, which can be obtained from Life Technologies, Inc. (Gaithersburg, Maryland).

A human genomic DNA library can be prepared by means well-known in the art (see, for example, Ausubel (1995) at pages 5-1 to 5-6; Wu (1997) at pages 307-327).

Genomic DNA can be isolated by lysing tissue with the detergent Sarkosyl, digesting the lysate with proteinase K, clearing insoluble debris from the lysate by centrifugation, precipitating nucleic acid from the lysate using isopropanol, and purifying resuspended DNA on a cesium chloride density gradient.

DNA fragments that are suitable for the production of a genomic library can be obtained by the random shearing of genomic DNA or by the partial digestion of genomic DNA with restriction endonucleases. Genomic DNA fragments can be inserted into a vector, such as a bacteriophage or cosmid vector, in accordance with conventional techniques, such as the use of restriction enzyme digestion to provide appropriate termini, the use of alkaline phosphatase treatment to avoid undesirable joining of DNA molecules, and ligation with appropriate ligases. Techniques for such manipulation are well-known in the art (see, for example, Ausubel (1995) at pages 5-1 to 5-6; Wu (1997) at pages 307-327).

Nucleic acid molecules that encode a TGF-beta binding-protein gene can also be obtained using the polymerase chain reaction (PCR) with oligonucleotide primers having nucleotide sequences that are based upon the nucleotide sequences of the human TGF-beta binding-protein gene, as described herein. General methods for screening libraries with PCR are provided by, for example, Yu et al., "Use of the

35

25

30

Polymerase Chain Reaction to Screen Phage Libraries," in Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications, White (ed.), pages 211-215 (Humana Press, Inc. 1993). Moreover, techniques for using PCR to isolate related genes are described by, for example, Preston, "Use of Degenerate Oligonucleotide Primers and the Polymerase Chain Reaction to Clone Gene Family Members," in Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications, White (ed.), pages 317-337 (Humana Press, Inc. 1993).

Alternatively, human genomic libraries can be obtained from commercial sources such as Research Genetics (Huntsville, AL) and the American Type Culture Collection (Rockville, Maryland).

A library containing cDNA or genomic clones can be screened with one or more polynucleotide probes based upon SEQ ID NO:1, using standard methods (see, for example, Ausubel (1995) at pages 6-1 to 6-11).

Anti-TGF-beta binding-protein antibodies, produced as described below, can also be used to isolate DNA sequences that encode TGF-beta binding-protein genes from cDNA libraries. For example, the antibodies can be used to screen \(\lambda\gamma11\) expression libraries, or the antibodies can be used for immunoscreening following hybrid selection and translation (see, for example, Ausubel (1995) at pages 6-12 to 6-16; Margolis et al., "Screening \(\lambda\) expression libraries with antibody and protein probes," in \(DNA\) Cloning 2: Expression Systems, 2nd Edition, Glover et al. (eds.), pages 1-14 (Oxford University Press 1995)).

The sequence of a TGF-beta binding-protein cDNA or TGF-beta binding-protein genomic fragment can be determined using standard methods. Moreover, the identification of genomic fragments containing a TGF-beta binding-protein promoter or regulatory element can be achieved using well-established techniques, such as deletion analysis (see, generally, Ausubel (1995)).

As an alternative, a TGF-beta binding-protein gene can be obtained by synthesizing DNA molecules using mutually priming long oligonucleotides and the nucleotide sequences described herein (see, for example, Ausubel (1995) at pages 8-8 to 8-9). Established techniques using the polymerase chain reaction provide the ability to synthesize DNA molecules at least two kilobases in length (Adang et al., *Plant Molec. Biol. 21*:1131, 1993; Bambot et al., *PCR Methods and Applications 2*:266, 1993; Dillon et al., "Use of the Polymerase Chain Reaction for the Rapid Construction of Synthetic Genes," in *Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications*, White (ed.), pages 263-268, (Humana Press, Inc. 1993); Holowachuk et al., *PCR Methods Appl. 4*:299, 1995).

3. Production of TGF-beta binding-protein genes

Nucleic acid molecules encoding variant TGF-beta binding-protein genes can be obtained by screening various cDNA or genomic libraries with polynucleotide probes having nucleotide sequences based upon SEQ ID NO:1, 5, 9, 11, 13, or 15, using procedures described above. TGF-beta binding-protein gene variants can also be constructed synthetically. For example, a nucleic acid molecule can be devised that encodes a polypeptide having a conservative amino acid change, compared with the amino acid sequence of SEQ ID NOs: 2, 6, 8, 10, 12, 14, or 16. That is, variants can be obtained that contain one or more amino acid substitutions of SEQ ID NOs: 2, 6, 8, 10, 12, 14 or 16, in which an alkyl amino acid is substituted for an alkyl amino acid in a TGF-beta binding-protein amino acid sequence, an aromatic amino acid is substituted for an aromatic amino acid in a TGF-beta binding-protein amino acid sequence, a sulfur-containing amino acid is substituted for a sulfur-containing amino acid in a TGF-beta binding-protein amino acid sequence, a hydroxy-containing amino acid is substituted for a hydroxy-containing amino acid in a TGF-beta binding-protein amino acid sequence, an acidic amino acid is substituted for an acidic amino acid in a TGF-beta binding-protein amino acid sequence, a basic amino acid is substituted for a basic amino acid in a TGF-beta binding-protein amino acid sequence, or a dibasic monocarboxylic amino acid is substituted for a dibasic monocarboxylic amino acid in a TGF-beta binding-protein amino acid sequence.

Among the common amino acids, for example, a "conservative amino acid substitution" is illustrated by a substitution among amino acids within each of the following groups: (1) glycine, alanine, valine, leucine, and isoleucine, (2) phenylalanine, tyrosine, and tryptophan, (3) serine and threonine, (4) aspartate and glutamate, (5) glutamine and asparagine, and (6) lysine, arginine and histidine. In making such substitutions, it is important to, where possible, maintain the cysteine backbone outlined in Figure 1.

Conservative amino acid changes in a TGF-beta binding-protein gene can be introduced by substituting nucleotides for the nucleotides recited in SEQ ID NO:1. Such "conservative amino acid" variants can be obtained, for example, by oligonucleotide-directed mutagenesis, linker-scanning mutagenesis, mutagenesis using the polymerase chain reaction, and the like (see Ausubel (1995) at pages 8-10 to 8-22; and McPherson (ed.), Directed Mutagenesis: A Practical Approach (IRL Press 1991)). The functional ability of such variants can be determined using a standard method, such as the assay described herein. Alternatively, a variant TGF-beta binding-protein polypeptide can be identified by the ability to specifically bind anti-TGF-beta binding-

10

15

protein antibodies.

Routine deletion analyses of nucleic acid molecules can be performed to obtain "functional fragments" of a nucleic acid molecule that encodes a TGF-beta binding-protein polypeptide. As an illustration, DNA molecules having the nucleotide sequence of SEQ ID NO:1 can be digested with *Bal*31 nuclease to obtain a series of nested deletions. The fragments are then inserted into expression vectors in proper reading frame, and the expressed polypeptides are isolated and tested for activity, or for the ability to bind anti-TGF-beta binding-protein antibodies. One alternative to exonuclease digestion is to use oligonucleotide-directed mutagenesis to introduce deletions or stop codons to specify production of a desired fragment. Alternatively, particular fragments of a TGF-beta binding-protein gene can be synthesized using the polymerase chain reaction.

Standard techniques for functional analysis of proteins are described by, for example, Treuter et al., *Molec. Gen. Genet. 240*:113, 1993; Content et al., "Expression and preliminary deletion analysis of the 42 kDa 2-5A synthetase induced by human interferon," in *Biological Interferon Systems, Proceedings of ISIR-TNO Meeting on Interferon Systems*, Cantell (ed.), pages 65-72 (Nijhoff 1987); Herschman, "The EGF Receptor," in *Control of Animal Cell Proliferation, Vol. 1*, Boynton et al., (eds.) pages 169-199 (Academic Press 1985); Coumailleau et al., *J. Biol. Chem.* 270:29270, 1995; Fukunaga et al., *J. Biol. Chem.* 270:25291, 1995; Yamaguchi et al., *Biochem. Pharmacol.* 50:1295, 1995; and Meisel et al., *Plant Molec. Biol.* 30:1, 1996.

The present invention also contemplates functional fragments of a TGF-beta binding-protein gene that have conservative amino acid changes.

A TGF-beta binding-protein variant gene can be identified on the basis of structure by determining the level of identity with nucleotide and amino acid 25 sequences of SEQ ID NOs: 1, 5, 9, 11, 13, or, 15 and 2, 6, 10, 12, 14, or 16, as discussed above. An alternative approach to identifying a variant gene on the basis of structure is to determine whether a nucleic acid molecule encoding a potential variant TGF-beta binding-protein gene can hybridize under stringent conditions to a nucleic acid molecule having the nucleotide sequence of SEQ ID Nos: 1, 5, 9, 11, 13, or, 15, or 30 a portion thereof of at least 15 or 20 nucleotides in length. As an illustration of stringent hybridization conditions, a nucleic acid molecule having a variant TGF-beta binding-protein sequence can bind with a fragment of a nucleic acid molecule having a sequence from SEQ ID NO:1 in a buffer containing, for example, 5xSSPE (1xSSPE = 180 mM sodium chloride, 10 mM sodium phosphate, 1 mM EDTA (pH 7.7), 35 5xDenhardt's solution (100xDenhardt's = 2% (w/v) bovine serum albumin, 2% (w/v)

15

10

15

20

25

Ficoll, 2% (w/v) polyvinylpyrrolidone) and 0.5% SDS incubated overnight at 55-60°C. Post-hybridization washes at high stringency are typically performed in 0.5xSSC (1xSSC = 150 mM sodium chloride, 15 mM trisodium citrate) or in 0.5xSSPE at 55-60 °C.

Regardless of the particular nucleotide sequence of a variant TGF-beta binding-protein gene, the gene encodes a polypeptide that can be characterized by its functional activity, or by the ability to bind specifically to an anti-TGF-beta binding-protein antibody. More specifically, variant TGF-beta binding-protein genes encode polypeptides which exhibit at least 50%, and preferably, greater than 60, 70, 80 or 90%, of the activity of polypeptides encoded by the human TGF-beta binding-protein gene described herein.

4. Production of TGF-beta binding-protein in Cultured Cells

To express a TGF-beta binding-protein gene, a nucleic acid molecule encoding the polypeptide must be operably linked to regulatory sequences that control transcriptional expression in an expression vector and then introduced into a host cell. In addition to transcriptional regulatory sequences, such as promoters and enhancers, expression vectors can include translational regulatory sequences and a marker gene which is suitable for selection of cells that carry the expression vector.

Expression vectors that are suitable for production of a foreign protein in eukaryotic cells typically contain (1) prokaryotic DNA elements coding for a bacterial replication origin and an antibiotic resistance marker to provide for the growth and selection of the expression vector in a bacterial host; (2) eukaryotic DNA elements that control initiation of transcription, such as a promoter; and (3) DNA elements that control the processing of transcripts, such as a transcription termination/polyadenylation sequence.

TGF-beta binding-proteins of the present invention are preferably expressed in mammalian cells. Examples of mammalian host cells include African green monkey kidney cells (Vero; ATCC CRL 1587), human embryonic kidney cells (293-HEK; ATCC CRL 1573), baby hamster kidney cells (BHK-21; ATCC CRL 8544), canine kidney cells (MDCK; ATCC CCL 34). Chinese hamster ovary cells (CHO-K1; ATCC CCL61), rat pituitary cells (GH1; ATCC CCL82), HeLa S3 cells (ATCC CCL2.2), rat hepatoma cells (H-4-II-E; ATCC CRL 1548) SV40-transformed monkey kidney cells (COS-1; ATCC CRL 1650) and murine embryonic cells (NIH-3T3; ATCC CRL 1658).

For a mammalian host, the transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, simian virus, or the like, in which the regulatory signals are associated with a particular gene

which has a high level of expression. Suitable transcriptional and translational regulatory sequences also can be obtained from mammalian genes, such as actin, collagen, myosin, and metallothionein genes.

Transcriptional regulatory sequences include a promoter region sufficient to direct the initiation of RNA synthesis. Suitable eukaryotic promoters include the promoter of the mouse metallothionein I gene [Hamer et al., J. Molec. Appl. Genet. 1:273, 1982], the TK promoter of Herpes virus [McKnight, Cell 31:355, 1982], the SV40 early promoter [Benoist et al., Nature 290:304, 1981], the Rous sarcoma virus promoter [Gorman et al., Proc. Nat'l Acad. Sci. USA 79:6777, 1982], the cytomegalovirus promoter [Foecking et al., Gene 45:101, 1980], and the mouse mammary tumor virus promoter (see, generally, 10 Etcheverry, "Expression of Engineered Proteins in Mammalian Cell Culture," in Protein Engineering: Principles and Practice, Cleland et al. (eds.), pages 163-181 (John Wiley & Sons, Inc. 1996)).

Alternatively, a prokaryotic promoter, such as the bacteriophage T3 RNA polymerase promoter, can be used to control TGF-beta binding-protein gene expression in mammalian cells if the prokaryotic promoter is regulated by a eukaryotic promoter (Zhou et al., Mol. Cell. Biol. 10:4529, 1990; Kaufman et al., Nucl. Acids Res. 19:4485, 1991).

TGF-beta binding-protein genes may also be expressed in bacterial, yeast, insect, or plant cells. Suitable promoters that can be used to express TGF-beta bindingprotein polypeptides in a prokaryotic host are well-known to those of skill in the art and 20 include promoters capable of recognizing the T4, T3, Sp6 and T7 polymerases, the $P_{\rm R}$ and P_L promoters of bacteriophage lambda, the trp, recA, heat shock, lacUV5, tac, lpplacSpr, phoA, and lacZ promoters of E. coli, promoters of B. subtilis, the promoters of the bacteriophages of Bacillus, Streptomyces promoters, the int promoter of bacteriophage lambda, the bla promoter of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene. Prokaryotic promoters have been reviewed by Glick, J. Ind. Microbiol. 1:277, 1987, Watson et al., Molecular Biology of the Gene, 4th Ed. (Benjamin Cummins 1987), and by Ausubel et al. (1995).

Preferred prokaryotic hosts include E. coli and Bacillus subtilus. Suitable strains of E. coli include BL21(DE3), BL21(DE3)pLysS, BL21(DE3)pLysE, 30 DH1, DH4I, DH5, DH5IF', DH5IMCR, DH10B, DH10B/p3, DH11S, C600, HB101, JM101, JM105, JM109, JM110, K38, RR1, Y1088, Y1089, CSH18, ER1451, and ER1647 (see, for example, Brown (Ed.), Molecular Biology Lahfax (Academic Press 1991)). Suitable strains of Bacillus subtilus include BR151, YB886, MI119, MI120, and B170 (see, for example, Hardy, "Bacillus Cloning Methods," in DNA 35 Cloning: A Practical Approach, Glover (Ed.) (IRL Press 1985)).

15

15

20

Methods for expressing proteins in prokaryotic hosts are well-known to those of skill in the art (see, for example, Williams et al., "Expression of foreign proteins in E. coli using plasmid vectors and purification of specific polyclonal antibodies," in DNA Cloning 2: Expression Systems, 2nd Edition, Glover et al. (eds.), page 15 (Oxford University Press 1995); Ward et al., "Genetic Manipulation and Expression of Antibodies," in Monoclonal Antibodies: Principles and Applications, page 137 (Wiley-Liss, Inc. 1995); and Georgiou, "Expression of Proteins in Bacteria," in Protein Engineering: Principles and Practice, Cleland et al. (eds.), page 101 (John Wiley & Sons, Inc. 1996)).

The baculovirus system provides an efficient means to introduce cloned TGF-heta hinding-protein genes into insect cells. Suitable expression vectors are based upon the Autographa californica multiple nuclear polyhedrosis virus (AcMNPV), and contain well-known promoters such as Drosophila heat shock protein (hsp) 70 promoter, Autographa californica nuclear polyhedrosis virus immediate-early gene promoter (ie-1) and the delayed early 39K promoter, baculovirus p10 promoter, and the Drosophila metallothionein promoter. Suitable insect host cells include cell lines derived from IPLB-Sf-21, a Spodopiera frugiperda pupal ovarian cell line, such as Sf9 (ATCC CRL 1711), Sf21AE, and Sf21 (Invitrogen Corporation; San Diego, CA), as well as Drosophila Schneider-2 cells. Established techniques for producing recombinant proteins in baculovirus systems are provided by Bailey et al., "Manipulation of Baculovirus Vectors," in Methods in Molecular Biology, Volume 7: Gene Transfer and Expression Protocols, Murray (ed.), pages 147-168 (The Humana Press, Inc. 1991), by Patel et al., "The baculovirus expression system," in DNA Cloning 2: Expression Systems, 2nd Edition, Glover et al. (eds.), pages 205-244 (Oxford University Press 1995), by Ausubel (1995) at pages 16-37 to 16-57, by Richardson (ed.), Baculovirus Expression Protocols (The Humana Press, Inc. 1995), and by Lucknow, "Insect Cell Expression Technology," in Protein Engineering: Principles and Practice, Cleland et al. (eds.), pages 183-218 (John Wiley & Sons, Inc. 1996).

Promoters for expression in yeast include promoters from GAL1 (galactose), PGK (phosphoglycerate kinase), ADH (alcohol dehydrogenase), AOX1 (alcohol oxidase), HIS4 (histidinol dehydrogenase), and the like. Many yeast cloning vectors have been designed and are readily available. These vectors include YIp-based vectors, such as YIp5, YRp vectors, such as YRp17, YEp vectors such as YEp13 and YCp vectors, such as YCp19. One skilled in the art will appreciate that there are a wide variety of suitable vectors for expression in yeast cells.

Expression vectors can also be introduced into plant protoplasts, intact plant

15

20

25

tissues, or isolated plant cells. General methods of culturing plant tissues are provided, for example, by Miki et al., "Procedures for Introducing Foreign DNA into Plants," in Methods in Plant Molecular Biology and Biotechnology, Glick et al. (eds.), pages 67-88 (CRC Press, 1993).

An expression vector can be introduced into host cells using a variety of standard techniques including calcium phosphate transfection. liposome-mediated transfection, microprojectile-mediated delivery, electroporation, and the like. Preferably, the transfected cells are selected and propagated to provide recombinant host cells that comprise the expression vector stably integrated in the host cell genome. Techniques for introducing vectors into eukaryotic cells and techniques for selecting such stable transformants using a dominant selectable marker are described, for example, by Ausubel (1995) and by Murray (ed.), Gene Transfer and Expression Protocols (Humana Press 1991). Methods for introducing expression vectors into bacterial, yeast, insect, and plant cells are also provided by Ausubel (1995).

General methods for expressing and recovering foreign protein produced by a mammalian cell system is provided by, for example, Etcheverry, "Expression of Engineered Proteins in Mammalian Cell Culture," in Protein Engineering: Principles and Practice, Cleland et al. (eds.), pages 163 (Wiley-Liss, Inc. 1996). Standard techniques for recovering protein produced by a bacterial system is provided by, for example, Grisshammer et al., "Purification of over-produced proteins from E. coli cells," in DNA Cloning 2: Expression Systems, 2nd Edition, Glover et al. (eds.), pages 59-92 (Oxford University Press 1995). Established methods for isolating recombinant proteins from a baculovirus system are described by Richardson (ed.), Baculovirus Expression Protocols (The Humana Press, Inc., 1995).

More generally, TGF-beta binding-protein can be isolated by standard techniques, such as affinity chromatography, size exclusion chromatography, ion exchange chromatography, HPLC and the like. Additional variations in TGF-beta binding-protein isolation and purification can be devised by those of skill in the art. For example, anti-TGF-beta binding-protein antibodies, obtained as described below, can be used to isolate large quantities of protein by immunoaffinity purification.

Production of Antibodies to TGF-beta binding-proteins 5.

Antibodies to TGF-beta binding-protein can be obtained, for example, using the product of an expression vector as an antigen. Particularly useful anti-TGFbeta binding-protein antibodies "bind specifically" with TGF-beta binding-protein of Sequence ID Nos. 2, 6, 10, 12, 14, or 16, but not to other TGF-beta binding-proteisn

15

20

25

30

such as Dan, Cerberus. SCGF, or Gremlin. Antibodies of the present invention (including fragments and derivatives thereof) may be a polyclonal or, especially a monoclonal antibody. The antibody may belong to any immunoglobulin class, and may be for example an IgG, for example IgG₁, IgG₂, IgG₃, IgG₄, IgE; IgM; or IgA antibody. It may be of animal, for example mammalian origin, and may be for example a murine, rat, human or other primate antibody. Where desired the antibody may be an internalising antibody.

Polyclonal antibodies to recombinant TGF-beta binding-protein can be prepared using methods well-known to those of skill in the art (see, for example, Green et al., "Production of Polyclonal Antisera," in *Immunochemical Protocols* (Manson, ed.), pages 1-5 (Humana Press 1992); Williams et al., "Expression of foreign proteins in *E. coli* using plasmid vectors and purification of specific polyclonal antibodies," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover et al. (eds.), page 15 (Oxford University Press 1995)). Although polyclonal antibodies are typically raised in animals such as rats, mice, rabbits, goats, or sheep, an anti-TGF-beta binding-protein antibody of the present invention may also be derived from a subhuman primate antibody. General techniques for raising diagnostically and therapeutically useful antibodies in baboons may be found, for example, in Goldenberg et al., international patent publication No. WO 91/11465 (1991), and in Losman et al., *Int. J. Cancer 46*:310, 1990.

The antibody should comprise at least a variable region domain. The variable region domain may be of any size or amino acid composition and will generally comprise at least one hypervariable amino acid sequence responsible for antigen binding embedded in a framework sequence. In general terms the variable (V) region domain may be any suitable arrangement of immunoglobulin heavy (V_H) and/or light (V_L) chain variable domains. Thus for example the V region domain may be monomeric and be a V_H or V_L domain where these are capable of independently binding antigen with acceptable affinity. Alternatively the V region domain may be dimeric and contain V_H - V_H , V_H - V_L , or V_L - V_L , dimers in which the V_H and V_L chains are non-covalently associated (abbreviated hereinafter as F_v). Where desired, however, the chains may be covalently coupled either directly, for example via a disulphide bond between the two variable domains, or through a linker, for example a peptide linker, to form a single chain domain (abbreviated hereinafter as scF_v).

The variable region domain may be any naturally occurring variable domain or an engineered version thereof. By engineered version is meant a variable region domain which has been created using recombinant DNA engineering techniques. Such engineered

versions include those created for example from natural antibody variable regions by insertions, deletions or changes in or to the amino acid sequences of the natural antibodies. Particular examples of this type include those engineered variable region domains containing at least one CDR and optionally one or more framework amino acids from one antibody and the remainder of the variable region domain from a second antibody.

The variable region domain may be covalently attached at a C-terminal amino acid to at least one other antibody domain or a fragment thereof. Thus, for example where a V_H domain is present in the variable region domain this may be linked to an immunoglobulin C_H 1 domain or a fragment thereof. Similarly a V_L domain may be linked to a C_K domain or a fragment thereof. In this way for example the antibody may be a Fab fragment wherein the antigen binding domain contains associated V_H and V_L domains covalently linked at their C-termini to a CH1 and C_K domain respectively. The CH1 domain may be extended with further amino acids, for example to provide a hinge region domain as found in a Fab' fragment, or to provide further domains, such as antibody CH2 and CH3 domains.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells (see, for example, Larrick et al., *Methods: A Companion to Methods in Enzymology 2:*106, 1991; Courtenay-Luck, "Genetic Manipulation of Monoclonal Antibodies," in *Monoclonal Antibodies: Production, Engineering and Clinical Application*, Ritter et al. (eds.), page 166 (Cambridge University Press 1995); and Ward et al., "Genetic Manipulation and Expression of Antibodies," in *Monoclonal Antibodies: Principles and Applications*, Birch et al., (eds.), page 137 (Wiley-Liss, Inc. 1995)).

Antibodies for use in the invention may in general be monoclonal (prepared by conventional immunisation and cell fusion procedures) or in the case of fragments, derived therefrom using any suitable standard chemical e.g. reduction or enzymatic cleavage and/or digestion techniques, for example by treatment with pepsin.

More specifically, monoclonal anti-TGF-beta binding-protein antibodies can be generated utilizing a variety of techniques. Rodent monoclonal antibodies to specific antigens may be obtained by methods known to those skilled in the art (see, for example, Kohler et al., *Nature 256*:495, 1975; and Coligan et al. (eds.), *Current Protocols in Immunology*; 1:2.5.1-2.6.7 (John Wiley & Sons 1991) ["Coligan"]; Picksley et al., "Production of monoclonal antibodies against proteins expressed in *E.*

15

25

30

15

20

25

coli," in DNA Cloning 2: Expression Systems, 2nd Edition, Glover et al. (eds.), page 93 (Oxford University Press 1995)).

Briefly, monoclonal antibodies can be obtained by injecting mice with a composition comprising a TGF-beta binding-protein gene product, verifying the presence of antibody production by removing a serum sample, removing the spleen to obtain B-lymphocytes, fusing the B-lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones which produce antibodies to the antigen, culturing the clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures.

In addition, an anti-TGF-beta binding-protein antibody of the present invention may be derived from a human monoclonal antibody. Human monoclonal antibodies are obtained from transgenic mice that have been engineered to produce specific human antibodies in response to antigenic challenge. In this technique, elements of the human heavy and light chain locus are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the endogenous heavy chain and light chain loci. The transgenic mice can synthesize human antibodies specific for human antigens, and the mice can be used to produce human antibody-secreting hybridomas. Methods for obtaining human antibodies from transgenic mice are described, for example, by Green et al., *Nature Genet.* 7:13, 1994; Lonberg et al., *Nature 368*:856, 1994; and Taylor et al., *Int. Immun. 6*:579, 1994.

Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography (see, for example, Coligan at pages 2.7.1-2.7.12 and pages 2.9.1-2.9.3; Baines et al., "Purification of Immunoglobulin G (IgG)," in Methods in Molecular Biology, Vol. 10, pages 79-104 (The Humana Press, Inc. 1992)).

For particular uses, it may be desirable to prepare fragments of anti-TGF-beta binding-protein antibodies. Such antibody fragments can be obtained, for example, by proteolytic hydrolysis of the antibody. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. As an illustration, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab')₂. This fragment can be further cleaved using a thiol reducing agent to produce 3.5S Fab' monovalent fragments. Optionally, the cleavage reaction can be performed using a blocking group for the sulfhydryl groups that result from cleavage of disulfide linkages. As an alternative, an enzymatic cleavage using pepsin produces two monovalent Fab

fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. patent No. 4,331,647, Nisonoff et al., *Arch Biochem. Biophys.* 89:230, 1960, Porter, *Biochem. J.* 73:119, 1959, Edelman et al., in *Methods in Enzymology* 1:422 (Academic Press 1967), and by Coligan at pages 2.8.1-2.8.10 and 2.10.-2.10.4.

Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

Alternatively, the antibody may be a recombinant or engineered antibody obtained by the use of recombinant DNA techniques involving the manipulation and reexpression of DNA encoding antibody variable and/or constant regions. Such DNA is known and/or is readily available from DNA libraries including for example phageantibody libraries (see Chiswell, D J and McCafferty, J. Tibtech. 10 80-84 (1992)) or where desired can be synthesised. Standard molecular biology and/or chemistry procedures may be used to sequence and manipulate the DNA, for example, to introduce codons to create cysteine residues, to modify, add or delete other amino acids or domains as desired.

From here, one or more replicable expression vectors containing the DNA may be prepared and used to transform an appropriate cell line, e.g. a non-producing myeloma cell line, such as a mouse NSO line or a bacterial, e.g. E.coli line, in which production of the antibody will occur. In order to obtain efficient transcription and translation, the DNA sequence in each vector should include appropriate regulatory sequences, particularly a promoter and leader sequence operably linked to the variable domain sequence. Particular methods for producing antibodies in this way are generally well known and routinely used. For example, basic molecular biology procedures are described by Maniatis et al (Molecular Cloning, Cold Spring Harbor Laboratory, New York, 1989); DNA sequencing can be performed as described in Sanger et al (PNAS 74, 5463, (1977)) and the Amersham International plc sequencing handbook; and site directed mutagenesis can be carried out according to the method of Kramer et al (Nucl. Acids Res. 12, 9441, (1984)) and the Anglian Biotechnology Ltd handbook. Additionally, there are numerous publications, detailing techniques suitable for the preparation of antibodies by manipulation of DNA, creation of expression vectors and transformation of appropriate cells, for example as reviewed by Mountain A and Adair, J R in Biotechnology and Genetic Engineering Reviews (ed. Tombs, M P, 10, Chapter 1, 1992, Intercept, Andover, UK) and in International Patent Specification No. WO 91/09967.

Where desired, the antibody according to the invention may have one or

10

20

25

15

more effector or reporter molecules attached to it and the invention extends to such modified proteins. The effector or reporter molecules may be attached to the antibody through any available amino acid side-chain, terminal amino acid or, where present carbohydrate functional group located in the antibody, always provided of course that this does not adversely affect the binding properties and eventual usefulness of the molecule. Particular functional groups include, for example any free amino, imino, thiol, hydroxyl, carboxyl or aldehyde group. Attachment of the antibody and the effector and/or reporter molecule(s) may be achieved via such groups and an appropriate functional group in the effector or reporter molecules. The linkage may be direct or indirect, through spacing or bridging groups.

Effector molecules include, for example, antineoplastic agents, toxins (such as enzymatically active toxins of bacterial or plant origin and fragments thereof e.g. ricin and fragments thereof) biologically active proteins, for example enzymes, nucleic acids and fragments thereof, e.g. DNA, RNA and fragments thereof, naturally occurring and synthetic polymers e.g. polysaccharides and polyalkylene polymers such as poly(ethylene glycol) and derivatives thereof, radionuclides, particularly radioiodide, and chelated metals. Suitable reporter groups include chelated metals, fluorescent compounds or compounds which may be detected by NMR or ESR spectroscopy.

Particular antineoplastic agents include cytotoxic and cytostatic agents, for example alkylating agents, such as nitrogen mustards (e.g. chlorambucil, melphalan, 20 mechlorethamine, cyclophosphamide, or uracil mustard) and derivatives thereof, triethylenephosphoramide, triethylenethiophosphor-amide, busulphan, cisplatin: antimetabolites, such as methotrexate, fluorouracil, floxuridine, cytarabine, mercaptopurine, thioguanine, fluoroacetic acid or fluorocitric acid, antibiotics, such as bleomycins (e.g. bleomycin sulphate), doxorubicin, daunorubicin, mitomycins (e.g. mitomycin C), 25 actinomycins (e.g. dactinomycin) plicamycin, calichaemicin and derivatives thereof, or esperamicin and derivatives thereof; mitotic inhibitors, such as etoposide, vincristine or vinblastine and derivatives thereof; alkaloids, such as ellipticine; polyols such as taxicin-I or taxicin-II; hormones, such as androgens (e.g. dromostanolone or testolactone), progestins (e.g. megestrol acetate or medroxyprogesterone acetate), estrogens (e.g. dimethylstilbestrol diphosphate, polyestradiol phosphate or estramustine phosphate) or antiestrogens (e.g. tamoxifen); anthraquinones, such as mitoxantrone, ureas, such as hydroxyurea; hydrazines, such as procarbazine; or imidazoles, such as dacarbazine.

Particularly useful effector groups are calichaemicin and derivatives thereof (see for example South African Patent Specifications Nos. 85/8794, 88/8127 and 90/2839).

Chelated metals include chelates of di-or tripositive metals having a

15

20

25

30

coordination number from 2 to 8 inclusive. Particular examples of such metals include technetium (Tc), rhenium (Re), cobalt (Co), copper (Cu), gold (Au), silver (Ag), lead (Pb), bismuth (Bi), indium (In), gallium (Ga), yttrium (Y), terbium (Tb), gadolinium (Gd), and scandium (Sc). In general the metal is preferably a radionuclide. Particular radionuclides include ^{99m}Tc, ¹⁸⁶Re, ¹⁸⁸Re, ⁵⁸Co, ⁶⁰Co, ⁶⁷Cu, ¹⁹⁵Au, ¹⁹⁹Au, ¹¹⁰Ag, ²⁰³Pb, ²⁰⁶Bi, ²⁰⁷Bi, ¹¹¹In, ⁶⁷Ga, ⁶⁸Ga, ⁸⁸Y, ⁹⁰Y, ¹⁶⁰Tb, ¹⁵³Gd and ⁴⁷Sc.

The chelated metal may be for example one of the above types of metal chelated with any suitable polydentate chelating agent, for example acyclic or cyclic polyamines, polyethers, (e.g. crown ethers and derivatives thereof); polyamides; porphyrins; and carbocyclic derivatives.

In general, the type of chelating agent will depend on the metal in use. One particularly useful group of chelating agents in conjugates according to the invention, however, are acyclic and cyclic polyamines, especially polyaminocarboxylic acids, for example diethylenetriaminepentaacetic acid and derivatives thereof, and macrocyclic amines, e.g. cyclic tri-aza and tetra-aza derivatives (for example as described in International Patent Specification No. WO 92/22583); and polyamides, especially desferrioxamine and derivatives thereof.

Thus for example when it is desired to use a thiol group in the antibody as the point of attachment this may be achieved through reaction with a thiol reactive group present in the effector or reporter molecule. Examples of such groups include an á-halocarboxylic acid or ester, e.g. iodoacetamide, an imide, e.g. maleimide, a vinyl sulphone, or a disulphide. These and other suitable linking procedures are generally and more particularly described in International Patent Specifications Nos. WO 93/06231, WO 92/22583, WO 90/091195 and WO 89/01476.

ASSAYS FOR SELECTING MOLECULES WHICH INCREASE BONE DENSITY

As discussed above, the present invention provides methods for selecting and/or isolating compounds which are capable of increasing bone density. For example, within one aspect of the present invention methods are provided for determining whether a selected molecule is capable of increasing bone mineral content, comprising the steps of (a) mixing a selected molecule with TGF-beta binding protein and a selected member of the TGF-beta family of proteins, (b) determining whether the selected molecule stimulates signaling by the TGF-beta family of proteins, or inhibits the binding of the TGF-beta binding protein to the TGF-beta family of proteins. Within certain embodiments, the molecule enhances the ability of TGF-beta to function as a positive regulator of mesenchymal cell differentiation.

Within other aspects of the invention, methods are provided for determining whether a selected molecule is capable of increasing bone mineral content, comprising the steps of (a) exposing a selected molecule to cells which express TGF-beta binding-protein and (b) determining whether the expression (or activity) of TGF-beta binding-protein from said exposed cells decreases, and therefrom determining whether the compound is capable of increasing bone mineral content. Within one embodiment, the cells are selected from the group consisting of the spontaneously transformed or untransformed normal human bone from bone biopsies and rat parietal bone osteoblasts. Such methods may be accomplished in a wide variety of assay formats including, for example, Countercurrent Immuno-Electrophoresis (CIEP), Radioimmunoassays, Radioimmunoprecipitations, Enzyme-Linked Immuno-Sorbent Assays (ELISA), Dot Blot assays, Inhibition or Competition assays, and sandwich assays (see U.S. Patent Nos. 4,376,110 and 4,486,530; see also Antibodies: A Laboratory Manual, supra).

Representative embodiments of such assays are provided below in Examples 5 and 6. Briefly, a family member of the TGF-beta super-family or a TGF-beta binding protein is first bound to a solid phase, followed by addition of a candidate molecule. The labeled family member of the TGF-beta super-family or a TGF-beta binding protein is then added to the assay, the solid phase washed, and the quantity of bound or labeled TGF-beta super-family member or TGF-beta binding protein on the solid support determined. Molecules which are suitable for use in increasing bone mineral content as described herein are those molecules which decrease the binding of TGF-beta binding protein to a member or members of the TGF-beta super-family in a statistically significant manner. Obviously, assays suitable for use within the present invention should not be limited to the embodiments described within Examples 2 and 3. In particular, numerous parameters may be altered, such as by binding TGF-beta to a solid phase, or by elimination of a solid phase entirely.

Within other aspects of the invention, methods are provided for determining whether a selected molecule is capable of increasing bone mineral content, comprising the steps of (a) exposing a selected molecule to cells which express TGF-beta and (b) determining whether the activity of TGF-beta from said exposed cells is altered, and therefrom determining whether the compound is capable of increasing bone mineral content. Similar to the above described methods, a wide variety of methods may be utilized to assess the changes of TGF-beta binding-protein expression due to a selected test compound.

15

20

25

30

25

For example, within one aspect of the present invention methods are provided for determining whether a selected molecule is capable of increasing bone mineral content, comprising the steps of (a) mixing a selected molecule with TGF-beta-binding-protein and a selected member of the TGF-beta family of proteins, (b) determining whether the selected molecule up-regulates the signaling of the TGF-beta family of proteins, or inhibits the binding of the TGF-beta binding-protein to the TGF-beta family of proteins. Within certain embodiments, the molecule enhances the ability of TGF-beta to function as a positive regulator of mechemchymal cell differentiation.

Similar to the above described methods, a wide variety of methods may be utilized to assess stimulation of TGF-beta due to a selected test compound. One such representative method is provided below in Example 6 (see also Durham et al., Endo. 136:1374-1380.

Within yet other aspects of the present invention, methods are provided for determining whether a selected molecule is capable of increasing bone mineral content, comprising the step of determining whether a selected molecule inhibits the binding of TGF-beta binding-protein to bone, or an analogue thereof. As utilized herein, it should be understood that bone or analogues thereof refers to hydroxyapatite, or a surface composed of a powdered form of bone, crushed bone or intact bone. Similar to the above described methods, a wide variety of methods may be utilized to assess the inhibition of TGF-beta binding-protein localization to bone matrix. One such representative method is provided below in Example 7.

It should be noted that while the methods recited herein may refer to the analysis of an individual test molecule, that the present invention should not be so limited. In particular, the selected molecule may be contained within a mixture of compounds. Hence, the recited methods may further comprise the step of isolating a molecule which inhibits the binding of TGF-beta binding-protein to a TGF-beta family member.

CANDIDATE MOLECULES

A wide variety of molecules may be assayed for their ability to inhibit the binding of TGF-beta binding-protein to a TGF-beta family member. Representative examples which are discussed in more detail below include organic molecules, proteins or peptides, and nucleic acid molecules. Although it should be evident from the discussion below that the candidate molecules described herein may be utilized in the

assays described herein, it should also be readily apparent that such molecules can also be utilized in a variety of diagnostic and therapeutic settins.

1. Organic Molecules

Numerous organic molecules may be assayed for their ability to inhibit the binding of TGF-beta binding-protein to a TGF-beta family member.

For example, within one embodiment of the invention suitable organic molecules may be selected from either a chemical library, wherein chemicals are assayed individually, or from combinatorial chemical libraries where multiple compounds are assayed at once, then deconvoluted to determine and isolate the most active compounds.

Representative examples of such combinatorial chemical libraries include those described by Agrafiotis et al., "System and method of automatically generating chemical compounds with desired properties," U.S. Patent No. 5,463,564; Armstrong, R.W., "Synthesis of combinatorial arrays of organic compounds through the use of multiple component combinatorial array syntheses," WO 95/02566; Baldwin, J.J. et al., "Sulfonamide derivatives and their use," WO 95/24186; Baldwin, J.J. et al., "Combinatorial dihydrobenzopyran library," WO 95/30642; Brenner, S., "New kit for preparing combinatorial libraries," WO 95/16918; Chenera, B. et al., "Preparation of library of resin-bound aromatic carbocyclic compounds," WO 95/16712; Ellman, J.A., "Solid phase and combinatorial synthesis of benzodiazepine compounds on a solid support," U.S. Patent No. 5,288,514; Felder, E. et al., "Novel combinatorial compound libraries," WO 95/16209; Lerner, R. et al., "Encoded combinatorial chemical libraries," WO 93/20242; Pavia, M.R. et al., "A method for preparing and selecting pharmaceutically useful non-peptide compounds from a structurally diverse universal library," WO 95/04277; Summerton, J.E. and D.D. Weller, "Morpholino-subunit combinatorial library and method," U.S. Patent No. 5,506,337; Holmes, C., "Methods for the Solid Phase Synthesis of Thiazolidinones, Metathiazanones, and Derivatives thereof," WO 96/00148; Phillips, G.B. and G.P. Wei, "Solid-phase Synthesis of Benzimidazoles," Tet. Letters 37:4887-90, 1996; Ruhland, B. et al., "Solid-supported Combinatorial Synthesis of Structurally Diverse \(\beta\)-Lactams," J. Amer. Chem. Soc. 111:253-4, 1996; Look, G.C. et al., "The Indentification of Cyclooxygenase-1 Inhibitors from 4-Thiazolidinone Combinatorial Libraries," Bioorg and Med. Chem. Letters 6:707-12, 1996.

10

20

25

20

25

30

Proteins and Peptides

A wide range of proteins and peptides may likewise be utilized as candidate molecules for inhibitors of the binding of TGF-beta binding-protein to a TGF-beta family member.

a. Combinatorial Peptide Libraries

Peptide molecules which are putative inhibitors of the binding of TGF-beta binding-protein to a TGF-beta family member may be obtained through the screening of combinatorial peptide libraries. Such libraries may either be prepared by one of skill in the art (see e.g., U.S. Patent Nos. 4,528,266 and 4,359,535, and Patent Cooperation Treaty Publication Nos. WO 92/15679, WO 92/15677, WO 90/07862, WO 90/02809, or purchased from commercially available sources (e.g., New England Biolabs Ph.D.TM Phage Display Peptide Library Kit).

b. Antibodies

Antibodies which inhibit the binding of TGF-beta binding-protein to a TGF-beta family member may readily be prepared given the disclosure provided herein. Within the context of the present invention, antibodies are understood to include monoclonal antibodies, polyclonal antibodies, anti-idiotypic antibodies, antibody fragments (e.g., Fab, and F(ab')₂, F_V variable regions, or complementarity determining regions). As discussed above, antibodies are understood to be specific against TGF-beta binding-protein, or against a specific TGF-beta family member, if they bind with a K_a of greater than or equal to 10⁷M, preferably greater than or equal to 10⁸M, and do not bind to other TGF-beta binding-proteins, or, bind with a K_a of less than or equal to 10⁶M. Furthermore, antibodies of the present invention should block or inhibit the binding of TGF-beta binding-protein to a TGF-beta family member.

The affinity of a monoclonal antibody or binding partner, as well as inhibition of binding can be readily determined by one of ordinary skill in the art (see Scatchard, Ann. N.Y. Acad. Sci. 51:660-672, 1949).

Briefly, polyclonal antibodies may be readily generated by one of ordinary skill in the art from a variety of warm-blooded animals such as horses, cows, various fowl, rabbits, mice, or rats. Typically, the TGF-beta binding-protein or unique peptide thereof of 13-20 amino acids (preferably conjugated to keyhole limpet hemocyanin by cross-linking with glutaraldehyde) is utilized to immunize the animal through intraperitoneal, intramuscular, intraocular, or subcutaneous injections, along with an adjuvant such as Freund's complete or incomplete adjuvant. Following several

25

30

35

booster immunizations, samples of serum are collected and tested for reactivity to the protein or peptide. Particularly preferred polyclonal antisera will give a signal on one of these assays that is at least three times greater than background. Once the titer of the animal has reached a plateau in terms of its reactivity to the protein, larger quantities of antisera may be readily obtained either by weekly bleedings, or by exsanguinating the animal.

Monoclonal antibodies may also be readily generated using conventional techniques (see U.S. Patent Nos. RE 32,011, 4,902,614, 4,543,439, and 4,411,993 which are incorporated herein by reference; see also Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses, Plenum Press, Kennett, McKearn, and Bechtol (eds.), 1980, and Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988, which are also incorporated herein by reference).

Briefly, within one embodiment a subject animal such as a rat or mouse is immunized with TGF-beta binding-protein or portion thereof as described above. The protein may be admixed with an adjuvant such as Freund's complete or incomplete adjuvant in order to increase the resultant immune response. Between one and three weeks after the initial immunization the animal may be reimmunized with another booster immunization, and tested for reactivity to the protein utilizing assays described above. Once the animal has reached a plateau in its reactivity to the injected protein, it is sacrificed, and organs which contain large numbers of B cells such as the spleen and lymph nodes are harvested.

Cells which are obtained from the immunized animal may be immortalized by infection with a virus such as the Epstein-Barr virus (EBV) (see Glasky and Reading, Hybridoma 8(4):377-389, 1989). Alternatively, within a preferred embodiment, the harvested spleen and/or lymph node cell suspensions are fused with a suitable myeloma cell in order to create a "hybridoma" which secretes monoclonal antibody. Suitable myeloma lines include, for example, NS-1 (ATCC No. TIB 18), and P3X63 - Ag 8.653 (ATCC No. CRL 1580).

Following the fusion, the cells may be placed into culture plates containing a suitable medium, such as RPMI 1640, or DMEM (Dulbecco's Modified Eagles Medium) (JRH Biosciences, Lenexa, Kansas), as well as additional ingredients, such as fetal bovine serum (FBS, *i.e.*, from Hyclone, Logan, Utah, or JRH Biosciences). Additionally, the medium should contain a reagent which selectively allows for the growth of fused spleen and myeloma cells such as HAT (hypoxanthine, aminopterin, and thymidine) (Sigma Chemical Co., St. Louis, Missouri). After about

15

20

25

30

35

seven days, the resulting fused cells or hybridomas may be screened in order to determine the presence of antibodies which are reactive against TGF-beta binding-protein (depending on the antigen used), and which block or inhibit the binding of TGF-beta binding-protein to a TGF-beta family member.

A wide variety of assays may be utilized to determine the presence of antibodies which are reactive against the proteins of the present invention, including for example countercurrent immuno-electrophoresis, radioimmunoassays, radioimmunoprecipitations, enzyme-linked immuno-sorbent assays (ELISA), dot blot assays, western blots, immunoprecipitation, inhibition or competition assays, and sandwich assays (see U.S. Patent Nos. 4,376,110 and 4,486,530; see also Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press. 1988). Following several clonal dilutions and reassays, a hybridoma producing antibodies reactive against the desired protein may be isolated.

Other techniques may also be utilized to construct monoclonal antibodies (see William D. Huse et al., "Generation of a Large Combinational Library of the Immunoglobulin Repertoire in Phage Lambda," Science 246:1275-1281, December 1989; see also L. Sastry et al., "Cloning of the Immunological Repertoire in Escherichia coli for Generation of Monoclonal Catalytic Antibodies: Construction of a Heavy Chain Variable Region-Specific cDNA Library," Proc. Natl. Acad. Sci. USA 86:5728-5732, August 1989; see also Michelle Alting-Mees et al., "Monoclonal Antibody Expression Libraries: A Rapid Alternative to Hybridomas," Strategies in Molecular Biology 3:1-9, January 1990). These references describe a commercial system available from Stratagene (La Jolla, California) which enables the production of antibodies through recombinant techniques. Briefly, mRNA is isolated from a B cell population, and utilized to create heavy and light chain immunoglobulin cDNA expression libraries in the $\lambda ImmunoZap(H)$ and $\lambda ImmunoZap(L)$ vectors. vectors may be screened individually or co-expressed to form Fab fragments or antibodies (see Huse et al., supra; see also Sastry et al., supra). Positive plaques may subsequently be converted to a non-lytic plasmid which allows high level expression of monoclonal antibody fragments from E. coli.

Similarly, portions or fragments, such as Fab and Fv fragments, of antibodies may also be constructed utilizing conventional enzymatic digestion or recombinant DNA techniques to incorporate the variable regions of a gene which encodes a specifically binding antibody. Within one embodiment, the genes which encode the variable region from a hybridoma producing a monoclonal antibody of interest are amplified using nucleotide primers for the variable region. These primers

may be synthesized by one of ordinary skill in the art, or may be purchased from commercially available sources. Stratagene (La Jolla, California) sells primers for mouse and human variable regions including, among others, primers for V_{Ha}, V_{Hb}, V_{Hc}, V_{Hd}, C_{H1}, V_L and C_L regions. These primers may be utilized to amplify heavy or light chain variable regions, which may then be inserted into vectors such as ImmunoZAPTM H or ImmunoZAPTM L (Stratagene), respectively. These vectors may then be introduced into *E. coli*, yeast, or mammalian-based systems for expression. Utilizing these techniques, large amounts of a single-chain protein containing a fusion of the V_H and V_L domains may be produced (see Bird et al., Science 2-12:423-426, 1988). In addition, such techniques may be utilized to change a "murine" antibody to a "human" antibody, without altering the binding specificity of the antibody.

Once suitable antibodies have been obtained, they may be isolated or purified by many techniques well known to those of ordinary skill in the art (see Amibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988). Suitable techniques include peptide or protein affinity columns, HPLC or RP-HPLC, purification on protein A or protein G columns, or any combination of these techniques.

c. Mutant TGF-beta binding-proteins

As described herein and below in the Examples (e.g., Examples 8 and 9), altered versions of TGF-beta binding-protein which compete with native TGF-beta binding-protein's ability to block the activity of a particular TGF-beta family member should lead to increased bone density. Thus, mutants of TGF-beta binding-protein which bind to the TGF-beta family member but do not inhibit the function of the TGF-beta family member would meet the criteria. The mutant versions must effectively compete with the endogenous inhibitory functions of TGF-beta binding-protein.

d. Production of proteins

Although various genes (or portions thereof) have been provided herein, it should be understood that within the context of the present invention, reference to one or more of these genes includes derivatives of the genes that are substantially similar to the genes (and, where appropriate, the proteins (including peptides and polypeptides) that are encoded by the genes and their derivatives). As used herein, a nucleotide sequence is deemed to be "substantially similar" if: (a) the nucleotide sequence is derived from the coding region of the above-described genes and includes, for example, portions of the sequence or allelic variations of the sequences discussed

20

25

30

35

above, or alternatively, encodes a molecule which inhibits the binding of TGF-beta binding-protein to a member of the TGF-beta family. (b) the nucleotide sequence is capable of hybridization to nucleotide sequences of the present invention under moderate, high or very high stringency (see Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, NY, 1989); or (c) the DNA sequences are degenerate as a result of the genetic code to the DNA sequences defined in (a) or (b). Further, the nucleic acid molecule disclosed herein includes both complementary and non-complementary sequences, provided the sequences otherwise meet the criteria set forth herein. Within the context of the present invention, high stringency means standard hybridization conditions (e.g., 5XSSPE, 0.5% SDS at 65°C, or the equivalent).

The structure of the proteins encoded by the nucleic acid molecules described herein may be predicted from the primary translation products using the hydrophobicity plot function of, for example, P/C Gene or Intelligenetics Suite (Intelligenetics, Mountain View, California), or according to the methods described by Kyte and Doolittle (*J. Mol. Biol. 15*~:105-132, 1982).

Proteins of the present invention may be prepared in the form of acidic or basic salts, or in neutral form. In addition, individual amino acid residues may be modified by oxidation or reduction. Furthermore, various substitutions, deletions, or additions may be made to the amino acid or nucleic acid sequences, the net effect of which is to retain or further enhance or decrease the biological activity of the mutant or wild-type protein. Moreover, due to degeneracy in the genetic code, for example, there may be considerable variation in nucleotide sequences encoding the same amino acid sequence.

Other derivatives of the proteins disclosed herein include conjugates of the proteins along with other proteins or polypeptides. This may be accomplished, for example, by the synthesis of N-terminal or C-terminal fusion proteins which may be added to facilitate purification or identification of proteins (see U.S. Patent No. 4,851,341, see also, Hopp et al., Bio/Technology 6:1204, 1988.) Alternatively, fusion proteins such as Flag/TGF-beta binding-protein be constructed in order to assist in the identification, expression, and analysis of the protein.

Proteins of the present invention may be constructed using a wide variety of techniques described herein. Further, mutations may be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following

ligation, the resulting reconstructed sequence encodes a derivative having the desired amino acid insertion, substitution, or deletion.

Alternatively, oligonucleotide-directed site-specific (or segment specific) mutagenesis procedures may be employed to provide an altered gene having particular codons altered according to the substitution, deletion, or insertion required. Exemplary methods of making the alterations set forth above are disclosed by Walder et al. (Gene 42:133, 1986); Bauer et al. (Gene 37:73, 1985); Craik (BioTechniques, January 1985, 12-19); Smith et al. (Genetic Engineering: Principles and Methods, Plenum Press, 1981); and Sambrook et al. (supra). Deletion or truncation derivatives of proteins (e.g., a soluble extracellular portion) may also be constructed by utilizing convenient restriction endonuclease sites adjacent to the desired deletion. Subsequent to restriction, overhangs may be filled in, and the DNA religated. Exemplary methods of making the alterations set forth above are disclosed by Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, 1989).

Mutations which are made in the nucleic acid molecules of the present invention preferably preserve the reading frame of the coding sequences. Furthermore, the mutations will preferably not create complementary regions that could hybridize to produce secondary mRNA structures, such as loops or hairpins, that would adversely affect translation of the mRNA. Although a mutation site may be predetermined, it is not necessary that the nature of the mutation per se be predetermined. For example, in order to select for optimum characteristics of mutants at a given site, random mutagenesis may be conducted at the target codon and the expressed mutants screened for indicative biological activity. Alternatively, mutations may be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes a derivative having the desired amino acid insertion, substitution, or deletion.

Nucleic acid molecules which encode proteins of the present invention may also be constructed utilizing techniques of PCR mutagenesis, chemical mutagenesis (Drinkwater and Klinedinst, *PNAS* 83:3402-3406, 1986), by forced nucleotide misincorporation (e.g., Liao and Wise Gene 88:107-111, 1990), or by use of randomly mutagenized oligonucleotides (Horwitz et al., Genome 3:112-117, 1989).

The present invention also provides for the manipulation and expression of the above described genes by culturing host cells containing a vector capable of expressing the above-described genes. Such vectors or vector constructs include either synthetic or cDNA-derived nucleic acid molecules encoding the desired protein, which

15

20

25

30

are operably linked to suitable transcriptional or translational regulatory elements. Suitable regulatory elements may be derived from a variety of sources, including bacterial, fungal, viral, mammalian, insect, or plant genes. Selection of appropriate regulatory elements is dependent on the host cell chosen, and may be readily accomplished by one of ordinary skill in the art. Examples of regulatory elements include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a transcriptional terminator, and a ribosomal binding sequence, including a translation initiation signal.

Nucleic acid molecules that encode any of the proteins described above may be readily expressed by a wide variety of prokaryotic and eukaryotic host cells, including bacterial, mammalian, yeast or other fungi, viral, insect, or plant cells. Methods for transforming or transfecting such cells to express foreign DNA are well known in the art (see, e.g., Itakura et al., U.S. Patent No. 4,704,362; Hinnen et al., Proc. Natl. Acad. Sci. USA 75:1929-1933, 1978; Murray et al., U.S. Patent No. 4,801,542; Upshall et al., U.S. Patent No. 4,935,349; Hagen et al., U.S. Patent No. 4,784,950; Axel et al., U.S. Patent No. 4,399,216; Goeddel et al., U.S. Patent No. 4,766,075; and Sambrook et al. Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, 1989; for plant cells see Czako and Marton, Plant Physiol. 104:1067-1071, 1994; and Paszkowski et al., Biotech. 24:387-392,

Bacterial host cells suitable for carrying out the present invention include $E.\ coli$, $B.\ subtilis$, $Salmonella\ nyphimurium$, and various species within the genera Pseudomonas, Streptomyces, and Staphylococcus, as well as many other bacterial species well known to one of ordinary skill in the art. Representative examples of bacterial host cells include DH5 α (Stratagene, LaJolla, California).

Bacterial expression vectors preferably comprise a promoter which functions in the host cell, one or more selectable phenotypic markers, and a bacterial origin of replication. Representative promoters include the β-lactamase (penicillinase) and lactose promoter system (see Chang et al., Nature 2-5:615, 1978), the T7 RNA polymerase promoter (Studier et al., Meth. Enzymol. 185:60-89, 1990), the lambda promoter (Elvin et al., Gene 8-123-126, 1990), the trp promoter (Nichols and Yanofsky, Meth. in Enzymology 101:155, 1983) and the tac promoter (Russell et al., Gene 20:231, 1982). Representative selectable markers include various antibiotic resistance markers such as the kanamycin or ampicillin resistance genes. Many plasmids suitable for transforming host cells are well known in the art, including among others, pBR322 (see Bolivar et al., Gene 2:95, 1977), the pUC plasmids pUC18,

25

25

pUC19, pUC118, pUC119 (see Messing, Meth. in Enzymology 101:20-77, 1983 and Vieira and Messing, Gene 19:259-268, 1982), and pNH8A, pNH16a, pNH18a, and Bluescript M13 (Stratagene, La Jolla, California).

Yeast and fungi host cells suitable for carrying out the present invention include, among others, Saccharomyces pombe, Saccharomyces cerevisiae, the genera Pichia or Khuyveromyces and various species of the genus Aspergillus (McKnight et al., U.S. Patent No. 4,935,349). Suitable expression vectors for yeast and fungi include, among others, YCp50 (ATCC No. 37419) for yeast, and the amdS cloning vector pV3 (Turnbull, Bio/Technology 7:169, 1989), YRp7 (Struhl et al., Proc. Natl. Acad. Sci. USA 76:1035-1039, 1978), YEp13 (Broach et al., Gene 8:121-133, 1979), pJDB249 and pJDB219 (Beggs, Nature 275:104-108, 1978) and derivatives thereof.

Preferred promoters for use in yeast include promoters from yeast glycolytic genes (Hitzeman et al., J. Biol. Chem. 255:12073-12080, 1980; Alber and Kawasaki, J. Mol. Appl. Genet. 1:419-434, 1982) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals, Hollaender 15 et al. (eds.), p. 355, Plenum, New York, 1982; Ammerer, Meth. Enzymol. 101:192-201, 1983). Examples of useful promoters for fungi vectors include those derived from Aspergillus nidulans glycolytic genes, such as the adh3 promoter (McKnight et al., EMBO J. 4:2093-2099, 1985). The expression units may also include a transcriptional terminator. An example of a suitable terminator is the adh3 terminator (McKnight 20 et al., ihid., 1985).

As with bacterial vectors, the yeast vectors will generally include a selectable marker, which may be one of any number of genes that exhibit a dominant phenotype for which a phenotypic assay exists to enable transformants to be selected. Preferred selectable markers are those that complement host cell auxotrophy, provide antibiotic resistance or enable a cell to utilize specific carbon sources, and include len2 (Broach et al., ibid.), ura3 (Botstein et al., Gene 8:17, 1979), or his3 (Struhl et al., Another suitable selectable marker is the cal gene, which confers chloramphenicol resistance on yeast cells.

30 Techniques for transforming fungi are well known in the literature, and have been described, for instance, by Beggs (ihid.), Hinnen et al. (Proc. Natl. Acad. Sci. USA 75:1929-1933, 1978), Yelton et al. (Proc. Natl. Acad. Sci. USA 81:1740-1747, 1984), and Russell (Nature 301:167-169, 1983). The genotype of the host cell may contain a genetic defect that is complemented by the selectable marker present on the expression vector. Choice of a particular host and selectable marker is well within the level of ordinary skill in the art.

Protocols for the transformation of yeast are also well known to those of ordinary skill in the art. For example, transformation may be readily accomplished either by preparation of spheroplasts of yeast with DNA (see Hinnen et al., PNAS USA 5:1929, 1978) or by treatment with alkaline salts such as LiCl (see Itoh et al., J. Bacteriology 153:163, 1983). Transformation of fungi may also be carried out using polyethylene glycol as described by Cullen et al. (Bio/Technology 5:369, 1987).

Viral vectors include those which comprise a promoter that directs the expression of an isolated nucleic acid molecule that encodes a desired protein as described above. A wide variety of promoters may be utilized within the context of the present invention, including for example, promoters such as MoMLV LTR, RSV LTR, Friend MuLV LTR, adenoviral promoter (Ohno et al., Science 265:781-784, 1994), neomycin phosphotransferase promoter/enhancer, late parvovirus promoter (Koering et al., Hum. Gene Therap. 5:457-463, 1994), Herpes TK promoter, SV40 promoter, metallothionein Ila gene enhancer/promoter, cytomegalovirus immediate early promoter, and the cytomegalovirus immediate late promoter. Within particularly preferred embodiments of the invention, the promoter is a tissue-specific promoter (see e.g., WO 91/02805; EP 0,415,731; and WO 90/07936). Representative examples of suitable tissue specific promoters include neural specific enolase promoter, platelet derived growth factor beta promoter, bone morphogenic protein promoter, human alpha1-chimaerin promoter, synapsin I promoter and synapsin II promoter. In addition to the above-noted promoters, other viral-specific promoters (e.g., retroviral promoters (including those noted above, as well as others such as HIV promoters), hepatitis, herpes (e.g., EBV), and bacterial, fungal or parasitic (e.g., malarial) -specific promoters may be utilized in order to target a specific cell or tissue which is infected with a virus, bacteria, fungus or parasite.

Mammalian cells suitable for carrying out the present invention include, among others COS, CHO, SaOS, osteosarcomas, KS483, MG-63, primary osteoblasts, and human or mammalian bone marrow stroma. Mammalian expression vectors for use in carrying out the present invention will include a promoter capable of directing the transcription of a cloned gene or cDNA. Preferred promoters include viral promoters and cellular promoters. Bone specific promoters include the bone sialo-protein and the promoter for osteocalcin. Viral promoters include the cytomegalovirus immediate early promoter (Boshart et al., Cell 41:521-530, 1985), cytomegalovirus immediate late promoter, SV40 promoter (Subramani et al., Mol. Cell. Biol. 1:854-864, 1981), MMTV LTR, RSV LTR, metallothionein-1, adenovirus E1a. Cellular promoters include the mouse metallothionein-1 promoter (Palmiter et al., U.S. Patent No. 4,579,821), a

15

20

25

30

Ю

15

mouse V_K promoter (Bergman et al., *Proc. Natl. Acad. Sci. USA 81*:7041-7045, 1983; Grant et al., *Nucl. Acids Res. 15*:5496, 1987) and a mouse V_H promoter (Loh et al., *Cell 33*:85-93, 1983). The choice of promoter will depend, at least in part, upon the level of expression desired or the recipient cell line to be transfected.

Such expression vectors may also contain a set of RNA splice sites located downstream from the promoter and upstream from the DNA sequence encoding the peptide or protein of interest. Preferred RNA splice sites may be obtained from adenovirus and/or immunoglobulin genes. Also contained in the expression vectors is a polyadenylation signal located downstream of the coding sequence of interest. Suitable polyadenylation signals include the early or late polyadenylation signals from SV40 (Kaufman and Sharp, *ihid.*), the polyadenylation signal from the Adenovirus 5 E1B region and the human growth hormone gene terminator (DeNoto et al., *Nuc. Acids Res.* 9:3719-3730, 1981). The expression vectors may include a noncoding viral leader sequence, such as the Adenovirus 2 tripartite leader, located between the promoter and the RNA splice sites. Preferred vectors may also include enhancer sequences, such as the SV40 enhancer. Expression vectors may also include sequences encoding the adenovirus VA RNAs. Suitable expression vectors can be obtained from commercial sources (e.g., Stratagene, La Jolla, California).

Vector constructs comprising cloned DNA sequences can be introduced into cultured mammalian cells by, for example, calcium phosphate-mediated 20 transfection (Wigler et al., Cell 14:725, 1978; Corsaro and Pearson, Somatic Cell Genetics 7:603, 1981; Graham and Van der Eb, Virology 52:456, 1973), electroporation (Neumann et al., EMBO J. 1:841-845, 1982), or DEAE-dextran mediated transfection (Ausubel et al. (eds.), Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY, 1987). To identify cells that have stably integrated the cloned DNA, a 25 selectable marker is generally introduced into the cells along with the gene or cDNA of interest. Preferred selectable markers for use in cultured mammalian cells include genes that confer resistance to drugs, such as neomycin, hygromycin, and methotrexate. The selectable marker may be an amplifiable selectable marker. Preferred amplifiable selectable markers are the DHFR gene and the neomycin resistance gene. Selectable 30 markers are reviewed by Thilly (Mammalian Cell Technology, Butterworth Publishers, Stoneham, Massachusetts, which is incorporated herein by reference).

Mammalian cells containing a suitable vector are allowed to grow for a period of time, typically 1-2 days, to begin expressing the DNA sequence(s) of interest.

Drug selection is then applied to select for growth of cells that are expressing the selectable marker in a stable fashion. For cells that have been transfected with an

15

amplifiable, selectable marker the drug concentration may be increased in a stepwise manner to select for increased copy number of the cloned sequences, thereby increasing expression levels. Cells expressing the introduced sequences are selected and screened for production of the protein of interest in the desired form or at the desired level. Cells that satisfy these criteria can then be cloned and scaled up for production.

Protocols for the transfection of mammalian cells are well known to those of ordinary skill in the art. Representative methods include calcium phosphate mediated transfection, electroporation, lipofection, retroviral, adenoviral and protoplast fusion-mediated transfection (see Sambrook et al., supra). Naked vector constructs can also be taken up by muscular cells or other suitable cells subsequent to injection into the muscle of a mammal (or other animals).

Numerous insect host cells known in the art can also be useful within the present invention, in light of the subject specification. For example, the use of baculoviruses as vectors for expressing heterologous DNA sequences in insect cells has been reviewed by Atkinson et al. (*Pestic. Sci. 28*:215-224,1990).

Numerous plant host cells known in the art can also be useful within the present invention, in light of the subject specification. For example, the use of *Agrobacterium rhizogenes* as vectors for expressing genes in plant cells has been reviewed by Sinkar et al. (*J. Biosci.* (*Bangalore*) 11:47-58, 1987).

Within related aspects of the present invention, proteins of the present 20 invention may be expressed in a transgenic animal whose germ cells and somatic cells contain a gene which encodes the desired protein and which is operably linked to a promoter effective for the expression of the gene. Alternatively, in a similar manner transgenic animals may be prepared that lack the desired gene (e.g., "knock-out" mice). Such transgenics may be prepared in a variety of non-human animals, including mice, 25 rats, rabbits, sheep, dogs, goats and pigs (see Hammer et al., Nature 315:680-683, 1985, Palmiter et al., Science 222:809-814, 1983, Brinster et al., Proc. Natl. Acad. Sci. USA 82:4438-4442, 1985, Palmiter and Brinster, Cell 41:343-345, 1985, and U.S. Patent Nos. 5,175,383, 5,087,571, 4,736,866, 5,387,742, 5,347,075, 5,221,778, and 5,175,384). Briefly, an expression vector, including a nucleic acid molecule to be 30 expressed together with appropriately positioned expression control sequences, is introduced into pronuclei of fertilized eggs, for example, by microinjection. Integration of the injected DNA is detected by blot analysis of DNA from tissue samples. It is preferred that the introduced DNA be incorporated into the germ line of the animal so that it is passed on to the animal's progeny. Tissue-specific expression may be achieved through the use of a tissue-specific promoter, or through the use of an

inducible promoter, such as the metallothionein gene promoter (Palmiter et al., 1983, ibid), which allows regulated expression of the transgene.

Proteins can be isolated by, among other methods, culturing suitable host and vector systems to produce the recombinant translation products of the present invention. Supernatants from such cell lines, or protein inclusions or whole cells where the protein is not excreted into the supernatant, can then be treated by a variety of purification procedures in order to isolate the desired proteins. For example, the supernatant may be first concentrated using commercially available protein concentration filters, such as an Amicon or Millipore Pellicon ultrafiltration unit. Following concentration, the concentrate may be applied to a suitable purification matrix such as, for example, an anti-protein antibody bound to a suitable support. Alternatively, anion or cation exchange resins may be employed in order to purify the protein. As a further alternative, one or more reverse-phase high performance liquid chromatography (RP-HPLC) steps may be employed to further purify the protein. Other methods of isolating the proteins of the present invention are well known in the skill of the art.

A protein is deemed to be "isolated" within the context of the present invention if no other (undesired) protein is detected pursuant to SDS-PAGE analysis followed by Coomassie blue staining. Within other embodiments, the desired protein can be isolated such that no other (undesired) protein is detected pursuant to SDS-PAGE analysis followed by silver staining.

3. Nucleic Acid Molecules

Within other aspects of the invention, nucleic acid molecules are provided which are capable of inhibiting TGF-beta binding-protein binding to a member of the TGF-beta family. For example, within one embodiment antisense oligonucleotide molecules are provided which specifically inhibit expression of TGF-beta binding-protein nucleic acid sequences (see generally, Hirashima et al. in Molecular Biology of RNA: New Perspectives (M. Inouye and B. S. Dudock, eds., 1987 Academic Press, San Diego, p. 401); Oligonucleotides: Antisense Inhibitors of Gene Expression (J.S. Cohen, ed., 1989 MacMillan Press, London); Stein and Cheng, Science 261:1004-1012, 1993; WO 95/10607; U.S. Patent No. 5,359,051; WO 92/06693; and EP-A2-612844). Briefly, such molecules are constructed such that they are complementary to, and able to form Watson-Crick base pairs with, a region of transcribed TGF-beta binding-protein mRNA sequence. The resultant double-stranded

15

20

25

nucleic acid interferes with subsequent processing of the mRNA, thereby preventing protein synthesis (see Example 10).

Within other aspects of the invention, ribozymes are provided which are capable of inhibiting the TGF-beta binding-protein binding to a member of the TGFbeta family. As used herein, "ribozymes" are intended to include RNA molecules that contain anti-sense sequences for specific recognition, and an RNA-cleaving enzymatic activity. The catalytic strand cleaves a specific site in a target RNA at greater than stoichiometric concentration. A wide variety of ribozymes may be utilized within the context of the present invention, including for example, the hammerhead ribozyme (for example, as described by Forster and Symons, Cell 48:211-220, 1987; Haseloff and Gerlach, Nature 328:596-600, 1988; Walbot and Bruening, Nature 334:196, 1988; Haseloff and Gerlach, Nature 334:585, 1988); the hairpin ribozyme (for example, as described by Haseloff et al., U.S. Patent No. 5,254,678, issued October 19, 1993 and Hempel et al., European Patent Publication No. 0 360 257, published March 26, 1990); and Tetrahymena ribosomal RNA-based ribozymes (see Cech et al., U.S. Patent No. 4,987,071). Ribozymes of the present invention typically consist of RNA, but may also be composed of DNA, nucleic acid analogs (e.g., phosphorothioates), or chimerics thereof (e.g., DNA/RNA/RNA).

4. Labels

The gene product or any of the candidate molecules described above and below, may be labeled with a variety of compounds, including for example, fluorescent molecules, toxins, and radionuclides. Representative examples of fluorescent molecules include fluorescein, *Phycobili* proteins, such as phycoerythrin, rhodamine, Texas red and luciferase. Representative examples of toxins include ricin, abrin diphtheria toxin, cholera toxin, gelonin, pokeweed antiviral protein, tritin, *Shigella* toxin, and *Pseudomonas* exotoxin A. Representative examples of radionuclides include Cu-64, Ga-67, Ga-68, Zr-89, Ru-97, Tc-99m, Rh-105, Pd-109, In-111, I-123, I-125, I-131, Re-186, Re-188, Au-198, Au-199, Pb-203, At-211, Pb-212 and Bi-212. In addition, the antibodies described above may also be labeled or conjugated to one partner of a ligand binding pair. Representative examples include avidin-biotin, and riboflavin-riboflavin binding protein.

Methods for conjugating or labeling the molecules described herein with the representative labels set forth above may be readily accomplished by one of ordinary skill in the art (see Trichothecene Antibody Conjugate, U.S. Patent No. 4,744,981; Antibody Conjugate, U.S. Patent No. 5,106,951; Fluorogenic Materials and

20

25

Labeling Techniques, U.S. Patent No. 4,018,884; Metal Radionuclide Labeled Proteins for Diagnosis and Therapy, U.S. Patent No. 4,897,255; and Metal Radionuclide Chelating Compounds for Improved Chelation Kinetics, U.S. Patent No. 4,988,496; see also Inman, Methods In Enzymology, Vol. 34, Affinity Techniques, Enzyme Purification: Part B, Jakoby and Wilchek (eds.), Academic Press, New York, p. 30, 1974; see also Wilchek and Bayer, "The Avidin-Biotin Complex in Bioanalytical Applications," Anal. Biochem. 171:1-32, 1988).

PHARMACEUTICAL COMPOSITIONS

As noted above, the present invention also provides a variety of pharmaceutical compositions, comprising one of the above-described molecules which inhibits the TGF-beta binding-protein binding to a member of the TGF-beta family along with a pharmaceutically or physiologically acceptable carrier, excipients or diluents. Generally, such carriers should be nontoxic to recipients at the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining the therapeutic agent with buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or dextrins, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with nonspecific serum albumin are exemplary appropriate diluents.

In addition, the pharmaceutical compositions of the present invention may be prepared for administration by a variety of different routes. In addition, pharmaceutical compositions of the present invention may be placed within containers, along with packaging material which provides instructions regarding the use of such pharmaceutical compositions. Generally, such instructions will include a tangible expression describing the reagent concentration, as well as within certain embodiments, relative amounts of excipient ingredients or diluents (e.g., water, saline or PBS) which may be necessary to reconstitute the pharmaceutical composition.

METHODS OF TREATMENT

The present invention also provides methods for increasing the mineral content and mineral density of bone. Briefly, numerous conditions result in the loss of bone mineral content, including for example, disease, genetic predisposition, accidents which result in the lack of use of bone (e.g., due to fracture), therapeutics which effect bone resorption, or which kill bone forming cells and normal aging. Through use of the molecules described herein which inhibit the TGF-beta binding-protein binding to a

TGF-beta family member such conditions may be treated or prevented. As utilized herein, it should be understood that bone mineral content has been increased, if bone mineral content has been increased in a statistically significant manner (v,g), greater than one-half standard deviation), at a selected site.

A wide variety of conditions which result in loss of bone mineral content may be treated with the molecules described herein. Patients with such conditions may be identified through clinical diagnosis utilizing well known techniques (see, e.g., Harrison's Principles of Internal Medicine, McGraw-Hill, Inc.). Representative examples of diseases that may be treated included dysplasias, wherein there is abnormal growth or development of bone. Representative examples of such conditions include achondroplasia, cleidocranial dysostosis, enchondromatosis, fibrous dysplasia, Gaucher's, hypophosphatemic rickets, Marfan's, multiple hereditary exotoses, neurofibromatosis, osteogenesis imperfecta, osteopetrosis, osteopoikilosis, sclerotic lesions, fractures, periodontal disease, pseudoarthrosis and pyogenic osteomyelitis.

Other conditions which may be treated or prevented include a wide variety of causes of osteopenia (i.e., a condition that causes greater than one standard deviation of bone mineral content or density below peak skeletal mineral content at youth). Representative examples of such conditions include anemic states, conditions caused steroids, conditions caused by heparin, bone marrow disorders, scurvy, malnutrition, calcium deficiency, idiopathic osteoporosis, congenital osteopenia or osteoporosis, alcoholism, chronic liver disease, senility, postmenopausal state, oligomenorrhea, amenorrhea, pregnancy, diabetes mellitus, hyperthyroidism, Cushing's disease, acromegaly, hypogonadism, immobilization or disuse, reflex sympathetic dystrophy syndrome, transient regional osteoporosis and osteomalacia.

Within one aspect of the present invention, bone mineral content or density may be increased by administering to a warm-blooded animal a therapeutically effective amount of a molecule which inhibits the TGF-beta binding-protein binding to a TGF-beta family member. Examples of warm-blooded animals that may be treated include both vertebrates and mammals, including for example horses, cows, pigs, sheep, dogs, cats, rats and mice. Representative examples of therapeutic molecules include ribozymes, ribozyme genes, antisense oligonucleotides and antibodies (e.g, humanized antibodies).

Within other aspects of the present invention, methods are provided for increasing bone density, comprising the step of introducing into cells which home to bone a vector which directs the expression of a molecule which inhibits the TGF-beta binding-protein binding to a member of the TGF-beta family, and administering the

15

25

vector containing cells to a warm-blooded animal. Briefly, cells which home to bone may be obtained directly from the bone of patients (e.g., cells obtained from the bone marrow such as CD34+, osteoblasts, osteocytes, and the like), from peripheral blood, or from cultures.

A vector which directs the expression of a molecule that inhibits the TGF-beta binding-protein binding to a member of the TGF-beta family is introduced into the cells. Representative examples of suitable vectors include viral vectors such as herpes viral vectors (e.g., U.S. Patent No. 5,288,641), adenoviral vectors (e.g., WO 94/26914, WO 93/9191; Kolls et al., PNAS 91(1):215-219, 1994; Kass-Eisler et al., PNAS 90(24):11498-502, 1993; Guzman et al., Circulation 88(6):2838-48, 1993; Guzman et al., Cir. Res. 73(6):1202-1207, 1993; Zabner et al., Cell 75(2):207-216, 1993; Li et al., Hum Gene Ther. 4(4):403-409, 1993; Caillaud et al., Eur. J. Neurosci. 5(10:1287-1291, 1993; Vincent et al., Nat. Genet. 5(2):130-134, 1993; Jaffe et al., Nat. Genet. 1(5):372-378, 1992; and Levrero et al., Gene 101(2):195-202, 1991), adenoassociated viral vectors (WO 95/13365; Flotte et al., PNAS 90(22):10613-10617, 1993), baculovirus vectors, parvovirus vectors (Koering et al., Hum. Gene Therap. 5:457-463, 1994), pox virus vectors (Panicali and Paoletti, PNAS 79:4927-4931, 1982; and Ozaki et al., Biochem. Biophys. Res. Comm. 193(2):653-660, 1993), and retroviruses (e.g., EP 0,415,731; WO 90/07936; WO 91/0285, WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent No. 5,219,740; WO 93/11230; WO 93/10218). Viral vectors may likewise be constructed which contain a mixture of different elements (e.g., promoters, envelope sequences and the like) from different viruses, or non-viral sources. Within various embodiments, either the viral vector itself, or a viral particle which contains the viral vector may be utilized in the methods and compositions described below.

Within other embodiments of the invention, nucleic acid molecules which encode a molecule which inhibits the TGF-beta binding-protein binding to a member of the TGF-beta family themselves may be administered by a variety of techniques, including, for example, administration of asialoosomucoid (ASOR) conjugated with poly-L-lysine DNA complexes (Cristano et al., PNAS 92122-92126, 1993), DNA linked to killed adenovirus (Curiel et al., Hum. Gene Ther. 3(2):147-154, 1992), cytofectin-mediated introduction (DMRIE-DOPE, Vical, California), direct DNA injection (Acsadi et al., Nature 352:815-818, 1991); DNA ligand (Wu et al., J. of Biol. Chem. 264:16985-16987, 1989); lipofection (Felgner et al., Proc. Natl. Acad. Sci. USA 84:7413-7417, 1989); liposomes (Pickering et al., Circ. 89(1):13-21, 1994; and Wang et al., PNAS 84:7851-7855, 1987); microprojectile bombardment (Williams

20

15

20

et al., *PNAS 88*:2726-2730, 1991); and direct delivery of nucleic acids which encode the protein itself either alone (Vile and Hart, *Cancer Res. 53*: 3860-3864, 1993), or utilizing PEG-nucleic acid complexes.

Representative examples of molecules which may be expressed by the vectors of present invention include ribozymes and antisense molecules, each of which are discussed in more detail above.

Determination of increased bone mineral content may be determined directly through the use of X-rays (e.g., Dual Energy X-ray Absorptometry or "DEXA"), or by inference through bone turnover markers (osteoblast specific alkaline phosphatase, osteocalcin, type 1 procollagen C' propeptide (PICP), and total alkaline phosphatase; see Comier, C., Curr. Opin. in Rheu. 7:243, 1995), or markers of bone resorption (pyridinoline, deoxypryridinoline, N-telopeptide, urinary hydroxyproline, plasma tartrate-resistant acid phosphatases and galactosyl hydroxylysine; see Comier, supra). The amount of bone mass may also be calculated from body weights, or utilizing other methods (see Guinness-Hey, Metab. Bone Dis. and Rel. Res. 5:177-181, 1984).

As will be evident to one of skill in the art, the amount and frequency of administration will depend, of course, on such factors as the nature and severity of the indication being treated, the desired response, the condition of the patient, and so forth. Typically, the compositions may be administered by a variety of techniques, as noted above.

The following examples are offered by way of illustration, and not by way of limitation.

20

25

30

EXAMPLES EXAMPLE I

SCLEROSTEOSIS MAPS TO THE LONG ARM OF HUMAN CHROMOSOME 17

Genetic mapping of the defect responsible for sclerosteosis in humans localized the gene responsible for this disorder to the region of human chromosome 17 that encodes a novel TGF-beta binding-protein family member. In sclerosteosis, skeletal bone displays a substantial increase in mineral density relative to that of unafflicted individuals. Bone in the head displays overgrowth as well. Sclerosteosis patients are generally healthy although they may exhibit variable degrees of syndactyly at birth and variable degrees of cranial compression and nerve compression in the skull.

Linkage analysis of the gene defect associated with sclerosteosis was conducted by applying the homozygosity mapping method to DNA samples collected from 24 South African Afrikaaner families in which the disease occurred. (Sheffield et al., 1994, *Human Molecular Genetics 3*:1331-1335. "Identification of a Bardet-Biedl syndrome locus on chromosome 3 and evaluation of an efficient approach to homozygosity mapping"). The Afrikaaner population of South Africa is genetically homogeneous; the population is descended from a small number of founders who colonized the area several centuries ago, and it has been isolated by geographic and social barriers since the founding. Sclerosteosis is rare everywhere in the world outside the Afrikaaner community, which suggests that a mutation in the gene was present in the founding population and has since increased in numbers along with the increase in the population. The use of homozygosity mapping is based on the assumption that DNA mapping markers adjacent to a recessive mutation are likely to be homozygous in affected individuals from consanguineous families and isolated populations.

A set of 371 microsatellite markers (Research Genetics, Set 6) from the autosomal chromosomes was selected to type pools of DNA from sclerosteosis patient samples. The DNA samples for this analysis came from 29 sclerosteosis patients in 24 families, 59 unaffected family members and a set of unrelated control individuals from the same population. The pools consisted of 4-6 individuals, either affected individuals, affected individuals from consanguineous families, parents and unaffected siblings, or unrelated controls. In the pools of unrelated individuals and in most of the pools with affected individuals or family members analysis of the markers showed several allele sizes for each marker. One marker, D17S1299, showed an indication of homozygosity: one band in several of the pools of affected individuals.

PCT/US99/27990

All 24 sclerosteosis families were typed with a total of 19 markers in the region of D17S1299 (at 17q12-q21). Affected individuals from every family were shown to be homozygous in this region, and 25 of the 29 individuals were homozygous for a core haplotype; they each had the same alleles between D17S1787 and D17S930. The other four individuals had one chromosome which matched this haplotype and a second which did not. In sum, the data compellingly suggested that this 3 megabase region contained the sclerosteosis mutation. Sequence analysis of most of the exons in this 3 megabase region identified a nonsense mutation in the novel TGF-beta binding-protein coding sequence (C>T mutation at position 117 of Sequence ID No. 1 results in a stop codon). This mutation was shown to be unique to sclerosteosis patients and carriers of Afrikaaner descent. The identity of the gene was further confirmed by identifying a mutation in its intron (A>T mutation at position +3 of the intron) which results in improper mRNA processing in a single, unrelated patient with diagnosed sclerosteosis.

15

10

EXAMPLE 2

TISSUE-SPECIFICITY OF TGF-BETA BINDING-PROTEIN GENE EXPRESSION
A. Human Beer Gene Expression by RT-PCR:

First-strand cDNA was prepared from the following total RNA samples using a commercially available kit ("Superscript Preamplification System for First-Strand cDNA Synthesis", Life Technologies, Rockville, MD): human brain, human liver, human spleen, human thymus, human placenta, human skeletal muscle, human thyroid, human pituitary, human osteoblast (NHOst from Clonetics Corp., San Diego, CA), human osteosarcoma cell line (Saos-2, ATCC# HTB-85), human bone, human bone marrow, human cartilage, vervet monkey bone, saccharomyces cerevisiae, and human peripheral blood monocytes. All RNA samples were purchased from a commercial source (Clontech, Palo Alto, CA), except the following which were prepared in-house: human osteoblast, human osteosarcoma cell line, human bone, human cartilage and vervet monkey bone. These in-house RNA samples were prepared using a commercially available kit ("TRI Reagent", Molecular Research Center, Inc., Cincinnati, OH).

PCR was performed on these samples, and additionally on a human genomic sample as a control. The sense Beer oligonucleotide primer had the sequence 5'-CCGGAGCTGGAGAACAACAAG-3' (SEQ ID NO:19) The antisense Beer oligonucleotide primer had the sequence 5'-GCACTGGCCGGAGCACACC-3' (SEQ

15

ID NO:20). In addition, PCR was performed using primers for the human beta-actin gene, as a control. The sense beta-actin oligonucleotide primer had the sequence 5'-AGGCCAACCGCGAGAAGATGA CC -3' (SEQ ID NO:21). The antisense beta-actin oligonucleotide primer had the sequence 5'-GAAGT CCAGGGCGACGTAGCA-3' (SEQ ID NO:22). PCR was performed using standard conditions in 25 ul reactions, with an annealing temperature of 61 degrees Celsius. Thirty-two cycles of PCR were performed with the Beer primers and twenty-four cycles were performed with the beta-actin primers.

Following amplification, 12 ul from each reaction were analyzed by agarose gel electrophoresis and ethidium bromide staining. See Figure 2A.

B. RNA In-situ Hybridization of Mouse Embryo Sections:

The full length mouse *Beer* cDNA (Sequence ID No. 11) was cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) in the antisense and sense direction using the manufacturer's protocol. ³⁵S-alpha-GTP-labeled cRNA sense and antisense transcripts were synthesized using in-vitro transcription reagents supplied by Ambion, Inc (Austin, TX). In-situ hybridization was performed according to the protocols of Lyons et al. (*J. Cell Biol. 111*:2427-2436, 1990).

The mouse Beer cRNA probe detected a specific message expressed in the neural tube, limb buds, blood vessels and ossifying cartilages of developing mouse 20 embryos. Panel A in Figure 3 shows expression in the apical ectodermal ridge (aer) of the limb (l) bud, blood vessels (bv) and the neural tube (nt). Panel B shows expression in the 4th ventricle of the brain (4). Panel C shows expression in the mandible (ma) cervical vertebrae (cv), occipital bone (oc), palate (pa) and a blood vessel (bv). Panel D shows expression in the ribs (r) and a heart valve (va). Panel A is a transverse 25 section of 10.5 dpc embryo. Panel B is a sagittal section of 12.5 dpc embryo and panels C and D are sagittal sections of 15.5 dpc embryos. ba=branchial arch, h=heart, te=telencephalon (forebrain), b=brain, f=frontonasal mass, g=gut, h=heart, j=jaw, li=liver, lu=lung, ot=otic vesicle, ao=, sc=spinal cord, skm=skeletal muscle, ns=nasal sinus, th=thymus , to=tongue, fl=forelimb, 30 di=diaphragm

EXAMPLE 3

35 EXPRESSION AND PURIFICATION OF RECOMBINANT BEER PROTEIN

A. Expression in COS-1 Cells:

15

The DNA sequence encoding the full length human Beer protein was amplified using the following PCR oligonucleotide primers: The 5' oligonucleotide primer had the sequence 5'-AAGCTTGGTACCATGCAGCTCCCAC-3' (SEQ ID NO:23) and contained a HindIII restriction enzyme site (in bold) followed by 19 nucleotides of the Beer gene starting 6 base pairs prior to the presumed amino terminal start codon (ATG). The 3' oligonucleotide primer had the sequence 5'-AAGCTTCTACTTGTCATCGTCGTCCT TGTAGTCGTAGGCGTTCTCCAGCT-3 (SEQ ID NO:24) and contained a HindIII restriction enzyme site (in bold) followed by a reverse complement stop codon (CTA) followed by the reverse complement of the FLAG epitope (underlined, Sigma-Aldrich Co., St. Louis, MO) flanked by the reverse complement of nucleotides coding for the carboxy terminal 5 amino acids of the Beer. The PCR product was TA cloned ("Original TA Cloning Kit", Invitrogen, Carlsbad, CA) and individual clones were screened by DNA sequencing. A sequence-verified clone was then digested by HindIII and purified on a 1.5% agarose gel using a commercially available reagents ("QIAquick Gel Extraction Kit", Qiagen Inc., Valencia, CA). This fragment was then ligated to HindIII digested, phosphatase-treated pcDNA3.1 (Invitrogen, Carlsbad, CA) plasmid with T4 DNA ligase. DH10B E. coli were transformed and plated on LB, $100~\mu g/ml$ ampicillin plates. Colonies bearing the desired recombinant in the proper orientation were identified by a PCR-based screen, using a 5' primer corresponding to the T7 promoter/priming site in pcDNA3.1 and a 3' primer with the sequence 5'- GCACTGGCCGGAGCACACC-3' (SEQ ID NO:25) that corresponds to the reverse complement of internal BEER sequence. The sequence of the cloned fragment was confirmed by DNA sequencing.

COS-1 cells (ATCC# CRL-1650) were used for transfection. 50 µg of the expression plasmid pcDNA-Beer-Flag was transfected using a commercially 25 available kit following protocols supplied by the manufacturer ("DEAE-Dextran Transfection Kit", Sigma Chemical Co., St. Louis, MO). The final media following transfection was DMEM (Life Technologies, Rockville, MD) containing 0.1% Fetal Bovine Serum. After 4 days in culture, the media was removed. Expression of recombinant BEER was analyzed by SDS-PAGE and Western Blot using anti-FLAG M2 monoclonal antibody (Sigma-Aldrich Co., St. Louis, MO). Purification of recombinant BEER protein was performed using an anti-FLAG M2 affinity column ("Mammalian Transient Expression System", Sigma-Aldrich Co., St. Louis, MO). The column profile was analyzed via SDS-PAGE and Western Blot using anti-FLAG M2 monoclonal antibody.

35

B. Expression in SF9 insect cells:

The human Beer gene sequence was amplified using PCR with standard conditions and the following primers:

Sense primer: 5'-GTCGTCGGATCCATGGGGTGGCAGGCGTTCAAGAATGAT-3' (SEQ ID NO:26)

Antisense primer: 5'-GTCGTCAAGCTTCTACTTGTCATCGTCCTTGTAGTCGTA GGCGTTCTCCAGCTCGGC-3' (SEQ ID NO:27)

The resulting cDNA contained the coding region of Beer with two modifications. The N-terminal secretion signal was removed and a FLAG epitope tag (Sigma) was fused in frame to the C-terminal end of the insert. BamHl and HindIII cloning sites were added and the gene was subcloned into pMelBac vector (Invitrogen) for transfer into a baculoviral expression vector using standard methods.

Recombinant baculoviruses expressing Beer protein were made using the Bac-N-Blue transfection kit (Invitrogen) and purified according to the manufacturers instructions.

SF9 cells (Invitrogen) were maintained in TNM_FH media (Invitrogen) containing 10% fetal calf serum. For protein expression, SF9 cultures in spinner flasks were infected at an MOI of greater than 10. Samples of the media and cells were taken daily for five days, and Beer expression monitored by western blot using an anti-FLAG M2 monoclonal antibody (Sigma) or an anti-Beer rabbit polyclonal antiserum.

After five days the baculovirus-infected SF9 cells were harvested by centrifugation and cell associated protein was extracted from the cell pellet using a high salt extraction buffer (1.5 M NaCl, 50 mM Tris pH 7.5). The extract (20 ml per 300 ml culture) was clarified by centrifugation, dialyzed three times against four liters of Tris buffered saline (150 mM NaCl, 50 mM Tris pH 7.5), and clarified by centrifugation again. This high salt fraction was applied to Hitrap Heparin (Pharmacia; 5 ml bed volume), washed extensively with HEPES buffered saline (25 mM HEPES 7.5, 150 mM Nacl) and bound proteins were eluted with a gradient from 150 mM NaCl to 1200 mM NaCl. Beer elution was observed at aproximately 800 mM NaCl. Beer containing fractions were supplemented to 10% glycerol and 1 mM DTT and frozen at -80 degrees C.

EXAMPLE 4

PREPARATION AND TESTING OF POLYCLONAL ANTIBODIES TO BEER, GREMLIN, AND DAN

15

20

25

30

WO 00/32773 PCT/US99/27990

A. Preparation of antigen:

The DNA sequences of Human Beer, Human Gremlin, and Human Dan were amplified using standard PCR methods with the following oligonucleotide primers:

5 H. Beer

Sense: 5'-GACTTGGATCCCAGGGGTGGCAGGCGTTC- 3' (SEQ ID NO:28) Antisense 5'-AGCATAAGCTTCTAGTAGGCGTTCTCCAG- 3' (SEQ ID NO:29) H. Gremlin

Sense: 5'-GACTTGGATCCGAAGGGAAAAAGAAAGGG-3' (SEQ ID NO:30)

Antisense: 5' -AGCATAAGCTTTTAATCCAAATCGATGGA- 3' (SEQ ID NO:31)

H. Dan

Sense: 5' -ACTACGAGCTCGGCCCCACCACCATCAACAAG- 3' (SEQ ID NO:32) Antisense: 5' -ACTTAGAAGCTTTCAGTCCTCAGCCCCCTCTTCC-3' (SEQ ID NO:33)

In each case the listed primers amplified the entire coding region minus the secretion signal sequence. These include restriction sites for subcloning into the bacterial expression vector pQE-30 (Qiagen Inc., Valencia, CA) at sites BamHI/HindIII for Beer and Gremlin, and sites SacI/HindIII for Dan. pQE30 contains a coding sequence for a 6x His tag at the 5' end of the cloning region. The completed constructs were transformed into *E. coli* strain M-15/pRep (Qiagen Inc) and individual clones verified by sequencing. Protein expression in M-15/pRep and purification (6xHis affinity tag binding to Ni-NTA coupled to Sepharose) were performed as described by the manufacturer (Qiagen, The QlAexpressionist).

The *E. coli*-derived Beer protein was recovered in significant quantity using solubilization in 6M guanidine and dialyzed to 2-4M to prevent precipitation during storage. Gremlin and Dan protein were recovered in higher quantity with solubilization in 6M guanidine and a post purification guanidine concentration of 0.5M.

B. Production and testing of polyclonal antibodies:

Polyclonal antibodies to each of the three antigens were produced in rabbit and in chicken hosts using standard protocols (R & R Antibody, Stanwood, WA; standard protocol for rabbit immunization and antisera recovery; Short Protocols in Molecular Biology. 2nd edition. 1992. 11.37-11.41. Contributors Helen M. Cooper and Yvonne Paterson; chicken antisera was generated with Strategic Biosolutions, Ramona, CA).

Rabbit antisera and chicken egg lgy fraction were screened for activity

via Western blot. Each of the three antigens was separated by PAGE and transferred to 0.45um nitrocellulose (Novex, San Diego, CA). The membrane was cut into strips with each strip containing approximately 75 ng of antigen. The strips were blocked in 3% Blotting Grade Block (Bio-Rad Laboratories, Hercules, CA) and washed 3 times in 1X Tris buffer saline (TBS) /0.02% TWEEN buffer. The primary antibody (preimmunization bleeds, rabbit antisera or chicken egg IgY in dilutions ranging from 1:100 to 1:10,000 in blocking buffer) was incubated with the strips for one hour with gentle rocking. A second series of three washes 1X TBS/0.02%TWEEN was followed by an one hour incubation with the secondary antibody (peroxidase conjugated donkey anti-rabbit, Amersham Life Science, Piscataway, NJ; or peroxidase conjugated donkey anti-chicken, Jackson ImmunoResearch, West Grove, PA). A final cycle of 3X washes of 1X TBS/0.02%TWEEN was performed and the strips were developed with Lumi-Light Western Blotting Substrate (Roche Molecular Biochemicals, Mannheim, Germany).

15

10

C. Antibody cross-reactivity test:

Following the protocol described in the previous section, nitrocellulose strips of Beer, Gremlin or Dan were incubated with dilutions (1:5000 and 1:10,000) of their respective rabbit antisera or chicken egg IgY as well as to antisera or chicken egg Igy (dilutions 1:1000 and 1:5000) made to the remaining two antigens. The increased levels of nonmatching antibodies was performed to detect low affinity binding by those antibodies that may be seen only at increased concentration. The protocol and duration of development is the same for all three binding events using the protocol described above. There was no antigen cross-reactivity observed for any of the antigens tested.

25

20

EXAMPLE 5

INTERACTION OF BEER WITH TGF-BETA SUPER-FAMILY PROTEINS

The interaction of Beer with proteins from different phylogenetic arms of the TGF-β superfamily were studied using immunoprecipitation methods. Purified TGFβ-1, TGFβ-2, TGFβ-3, BMP-4, BMP-5, BMP-6 and GDNF were obtained from commerical sources (R&D systems; Minneapolis, MN). A representative protocol is as follows. Partially purified Beer was dialyzed into HEPES buffered saline (25 mM HEPES 7.5, 150 mM NaCl). Immunoprecipitations were done in 300 ul of IP buffer (150 mM NaCl, 25 mM Tris pH 7.5, 1mM EDTA, 1.4 mM β-mercaptoethanol, 0.5 % triton X 100, and 10% glycerol). 30 ng recombinant human BMP-5 protein (R&D

20

25

systems) was applied to 15 ul of FLAG affinity matrix (Sigma; St Louis MO)) in the presence and absence of 500 ng FLAG epitope-tagged Beer. The proteins were incubated for 4 hours @ 4°Cand then the affinity matrix-associated proteins were washed 5 times in IP buffer (1 ml per wash). The bound proteins were eluted from the affinity matrix in 60 microliters of 1X SDS PAGE sample buffer. The proteins were resolved by SDS PAGE and Beer associated BMP-5 was detected by western blot using anti-BMP-5 antiserum (Research Diagnostics, Inc) (see Figure 5).

BEER Ligand Binding Assay:

FLAG-Beer protein (20 ng) is added to 100 ul PBS/0.2% BSA and adsorbed into each well of 96 well microtiter plate previously coated with anti-FLAG monoclonal antibody (Sigma; St Louis MO) and blocked with 10% BSA in PBS. This is conducted at room temperature for 60 minutes. This protein solution is removed and the wells are washed to remove unbound protein. BMP-5 is added to each well in concentrations ranging from10 pM to 500 nM in PBS/0.2% BSA and incubated for 2 hours at room temperature. The binding solution is removed and the plate washed with three times with 200ul volumes of PBS/0.2% BSA. BMP-5 levels are then detected using BMP-5 anti-serum via ELISA (F.M. Ausubel et al (1998) Current Protocols in Mol Biol. Vol 2 11.2.1-11.2.22). Specific binding is calculated by subtracting non-specific binding from total binding and analyzed by the LIGAND program (Munson and Podbard, Anal. Biochem., 107, p220-239, (1980).

In a variation of this method, Beer is engineered and expressed as a human Fc fusion protein. Likewise the ligand BMP is engineered and expressed as mouse Fc fusion. These proteins are incubated together and the assay conducted as described by Mellor et al using homogeneous time resolved fluorescence detection (G.W. Mellor et al., *J of Biomol Screening*, 3(2) 91-99, 1998).

EXAMPLE 6

SCREENING ASSAY FOR INHIBITION OF TGF-BETA BINDING-PROTEIN
BINDING TO TGF-BETA FAMILY MEMBERS

The assay described above is replicated with two exceptions. First, BMP concentration is held fixed at the Kd determined previously. Second, a collection of antagonist candidates is added at a fixed concentration (20 uM in the case of the small organic molecule collections and 1 uM in antibody studies). These candidate molecules (antagonists) of TGF-beta binding-protein binding include organic

compounds derived from commercial or internal collections representing diverse chemical structures. These compounds are prepared as stock solutions in DMSO and are added to assay wells at ≤ 1% of final volume under the standard assay conditions. These are incubated for 2 hours at room temperature with the BMP and Beer, the solution removed and the bound BMP is quantitated as described. Agents that inhibit 40% of the BMP binding observed in the absence of compound or antibody are considered antagonists of this interaction. These are further evaluated as potential inhibitors based on titration studies to determine their inhibition constants and their influence on TGF-beta binding-protein binding affinity. Comparable specificity control assays may also be conducted to establish the selectivity profile for the identified antagonist through studies using assays dependent on the BMP ligand action (e.g. BMP/BMP receptor competition study).

15

20

25

EXAMPLE 7

INHIBITION OF TGF-BETA BINDING-PROTEIN LOCALIZATION TO BONE MATRIX

Evaluation of inhibition of localization to bone matrix (hydroxyapatite) is conducted using modifications to the method of Nicolas (Nicolas, V. Calcif Tissue Int 5⁻:206, 1995). Briefly, ¹²⁵I-labelled TGF-beta binding-protein is prepared as described by Nicolas (supra). Hydroxyapatite is added to each well of a 96 well microtiter plate equipped with a polypropylene filtration membrane (Polyfiltroninc, Weymouth MA). TGF-beta binding-protein is added to 0.2% albumin in PBS buffer. The wells containing matrix are washed 3 times with this buffer. Adsorbed TGF-beta binding-protein is eluted using 0.3M NaOH and quantitated.

Inhibitor identification is conducted via incubation of TGF-beta binding-protein with test molecules and applying the mixture to the matrix as described above. The matrix is washed 3 times with 0.2% albumin in PBS buffer. Adsorbed TGF-beta binding-protein is eluted using 0.3 M NaOH and quantitated. Agents that inhibit 40% of the TGF-beta binding-protein binding observed in the absence of compound or antibody are considered bone localization inhibitors. These inhibitors are further characterized through dose response studies to determine their inhibition constants and their influence on TGF-beta binding-protein binding affinity.

EXAMPLE 8

CONSTRUCTION OF TGF-BETA BINDING-PROTEIN MUTANT

A. Mutagenesis:

A full-length TGF-beta binding-protein cDNA in pBluescript SK serves as a template for mutagenesis. Briefly, appropriate primers (see the discussion provided above) are utilized to generate the DNA fragment by polymerase chain reaction using Vent DNA polymerase (New England Biolabs, Beverly, MA). The polymerase chain reaction is run for 23 cycles in buffers provided by the manufacturer using a 57°C annealing temperature. The product is then exposed to two restriction enzymes and after isolation using agarose gel electrophoresis, ligated back into pRBP4-503 from which the matching sequence has been removed by enzymatic digestion. Integrity of the mutant is verified by DNA sequencing.

B. Mammalian Cell Expression and Isolation of Mutant TGF-beta binding-protein:

The mutant TGF-beta binding-protein cDNAs are transferred into the pcDNA3.1 mammalian expression vector described in EXAMPLE 3. After verifying the sequence, the resultant constructs are transfected into COS-1 cells, and secreted protein is purified as described in EXAMPLE 3.

20

15

5

EXAMPLE 9

ANIMAL MODELS -I

GENERATION OF TRANSGENIC MICE OVEREXPRESSING THE BEER GENE

25 genomic DNA library (distributed by Research Genetics, Huntsville, AL) was used to determine the complete sequence of the mouse *Beer* gene and its 5' and 3' flanking regions. A 41 kb SalI fragment, containing the entire gene body, plus ~17 kb of 5' flanking and ~20 kb of 3' flanking sequence was sub-cloned into the BamHI site of the SuperCosl cosmid vector (Stratagene, La Jolla, CA) and propagated in the *E. coli* strain DH10B. From this cosmid construct, a 35 kb MluI - AviII restriction fragment (Sequence No. 6), including the entire mouse *Beer* gene, as well as 17 kb and 14 kb of 5' and 3' flanking sequence, respectively, was then gel purified, using conventional means, and used for microinjection of mouse zygotes (DNX Transgenics; US Patent No. 4,873,191). Founder animals in which the cloned DNA fragment was integrated randomly into the genome were obtained at a frequency of 5-30% of live-born pups. The presence of the transgene was ascertained by performing Southern blot analysis of

genomic DNA extracted from a small amount of mouse tissue, such as the tip of a tail DNA was extracted using the following protocol: tissue was digested overnight at 55° C in a lysis buffer containing 200 mM NaCl, 100 mM Tris pH8.5, 5 mM EDTA, 0.2% SDS and 0.5 mg/ml Proteinase K. The following day, the DNA was extracted once with phenol/chloroform (50:50), once with chloroform/isoamylalcohol (24:1) and precipitated with ethanol. Upon resuspension in TE (10mM Tris pH7.5, 1 mM EDTA) 8-10 ug of each DNA sample were digested with a restriction endonuclease, such as EcoRI, subjected to gel electrophoresis and transferred to a charged nylon membrane, such as HyBondN+ (Amersham, Arlington Heights, IL). The resulting filter was then hybridized with a radioactively labelled fragment of DNA deriving from the mouse Beer gene locus, and able to recognize both a fragment from the endogenous gene locus and a fragment of a different size deriving from the transgene. Founder animals were bred to normal non-transgenic mice to generate sufficient numbers of transgenic and non-transgenic progeny in which to determine the effects of Beer gene overexpression. For these studies, animals at various ages (for example, 1 day, 3 weeks, 6 weeks, 4 months) are subjected to a number of different assays designed to ascertain gross skeletal formation, bone mineral density, bone mineral content, osteoclast and osteoblast activity, extent of endochondral ossification, cartilage formation, etc. The transcriptional activity from the transgene may be determined by extracting RNA from various tissues, and using an RT-PCR assay which takes advantage of single nucleotide polymorphisms between the mouse strain from which the transgene is derived (129Sv/J) and the strain of mice used for DNA microinjection [(C57BL5/J x SJL/J)F2].

ANIMAL MODELS - II

DISRUPTION OF THE MOUSE BEER GENE BY HOMOLOGOUS RECOMBINATION

Homologous recombination in embryonic stem (ES) cells can be used to inactivate the endogenous mouse *Beer* gene and subsequently generate animals carrying the loss-of-function mutation. A reporter gene, such as the *E. coli* β -galactosidase gene, was engineered into the targeting vector so that its expression is controlled by the endogenous *Beer* gene's promoter and translational initiation signal. In this way, the spatial and temporal patterns of *Beer* gene expression can be determined in animals carrying a targeted allele.

The targeting vector was constructed by first cloning the drug-selectable phosphoglycerate kinase (PGK) promoter driven neomycin-resistance gene (neo) cassette from pGT-N29 (New England Biolabs, Beverly, MA) into the cloning vector pSP72 (Promega, Madson, WI). PCR was used to flank the PGKneo cassette with

15

20

25

30

35

bacteriophage P1 loxP sites, which are recognition sites for the P1 Cre recombinase (Hoess et al., PNAS USA, 79:3398, 1982). This allows subsequent removal of the neoresistance marker in targeted ES cells or ES cell-derived animals (US Patent 4,959,317). The PCR primers were comprised of the 34 nucleotide (ntd) loxP sequence, 15-25 ntd complementary to the 5' and 3' ends of the PGKneo cassette, as well as restriction enzyme recognition sites (BamHI in the sense primer and EcoRI in the anti-sense primer) for cloning into pSP72. The sequence of the sense primer was 5'-

AATCTGGATCCATAACTTCGTATAGCATACATTATACGAAGTTATCTGCAG GATTCGAGGGCCCCT-3' (SEQ ID NO:34); sequence of the anti-sense primer was 5'-AATCTGAATTCCACCGGTGTTAATTAAATAACTTCGT ATAATGTATGCTATACGAAGTTATAGATCTAGAG TCAGCTTCTGA-3' (SEQ ID NO:35).

The next step was to clone a 3.6 kb Xhol-HindllI fragment, containing the E. coli β -galactosidase gene and SV40 polyadenylation signal from pSV β (Clontech, Palo Alto, CA) into the pSP72-PGKneo plasmid. The "short arm" of homology from the mouse Beer gene locus was generated by amplifying a 2.4 kb fragment from the BAC clone 15G5. The 3' end of the fragment coincided with the translational initiation site of the Beer gene, and the anti-sense primer used in the PCR also included 30 ntd complementary to the 5' end of the β -galactosidase gene so that its 20 coding region could be fused to the Beer initiation site in-frame. The approach taken for introducing the "short arm" into the pSP72-βgal-PGKneo plasmid was to linearize the plasmid at a site upstream of the β -gal gene and then to co-transform this fragment with the "short arm" PCR product and to select for plasmids in which the PCR product was integrated by homologous recombination. The sense primer for the "short arm" 25 amplification included 30 ntd complementary to the pSP72 vector to allow for this recombination event. The sequence of the sense primer was 5'-ATTTAGGTGACACT ATAGAACTCGAGCAGCTGAAGCTTAACCACATGGTGGCTCACAACCAT-3' (SEQ ID NO:36) and the sequence of the anti-sense primer was 5'-AACGACGCCAGTGAATCCGTA

ATCATGGTCATGCTGCCAGGTGGAGGAGGGCA-3' (SEQ ID NO:37).

The "long arm" from the Beer gene locus was generated by amplifying a 6.1 kb fragment from BAC clone 15G5 with primers which also introduce the rarecutting restriction enzyme sites SgrAI, Fsel, Ascl and PacI. Specifically, the sequence of the sense primer was 5'-ATTACCACCGGTGACACCCGCTTCCTGACAG-3' (SEQ ID NO:38); the sequence of the anti-sense primer 5'-

10

15

20

ATTACTTAATTAAACATGGCGCGCCAT

ATGGCCGGCCCCTAATTGCGGCGCATCGTTAATT-3' (SEQ ID NO:39). The resulting PCR product was cloned into the TA vector (Invitrogen, Carlsbad, CA) as an intermediate step.

The mouse Beer gene targeting construct also included a second selectable marker, the herpes simplex virus I thymidine kinase gene (HSVTK) under the control of rous sarcoma virus long terminal repeat element (RSV LTR). Expression of this gene renders mammalian cells sensitive (and inviable) to gancyclovir; it is therefore a convenient way to select against neomycin-resistant cells in which the construct has integrated by a non-homologous event (US Patent 5,464,764). The RSVLTR-HSVTK cassette was amplified from pPS1337 using primers that allow subsequent cloning into the Fsel and Ascl sites of the "long arm"-TA vector plasmid. this PCR. the sequence of the sense primer ATTACGGCCGCCAAAGGAATTCAAGA TCTGA-3' (SEQ ID NO:40); the sequence of the anti-sense primer 5'-ATTACGGCGCCCCCTC was ACAGGCCGCACCCAGCT-3' (SEQ ID NO:41).

The final step in the construction of the targeting vector involved cloning the 8.8 kb SgrAI-AscI fragment containing the "long arm" and RSVLTR-HSVTK gene into the SgrAI and AscI sites of the pSP72-"short arm"-\betagal-PGKneo plasmid. This targeting vector was linearized by digestion with either AscI or PacI before electroporation into ES cells.

EXAMPLE 10

25

30

35

ANTISENSE-MEDIATED BEER INACTIVATION

17-nucleotide antisense oligonucleotides are prepared in an overlapping format, in such a way that the 5' end of the first oligonucleotide overlaps the translation initiating AUG of the Beer transcript, and the 5' ends of successive oligonucleotides occur in 5 nucleotide increments moving in the 5' direction (up to 50 nucleotides away), relative to the Beer AUG. Corresponding control oligonucleotides are designed and prepared using equivalent base composition but redistributed in sequence to inhibit any significant hybridization to the coding mRNA. Reagent delivery to the test cellular system is conducted through cationic lipid delivery (P.L. Felgner, *Proc. Natl. Acad. Sci. USA 84*:7413, 1987). 2 ug of antisense oligonucleotide is added to 100 ul of reduced serum media (Opti-MEM 1 reduced serum media; Life Technologies, Gaithersburg MD) and this is mixed with Lipofectin reagent (6 ul) (Life Technologies,

15

Gaithersburg MD) in the 100 ul of reduced serum media. These are mixed, allowed to complex for 30 minutes at room temperature and the mixture is added to previously seeded MC3T3E21 or KS483 cells. These cells are cultured and the mRNA recovered. Beer mRNA is monitored using RT-PCR in conjunction with Beer specific primers. In addition, separate experimental wells are collected and protein levels characterized through western blot methods described in Example 4. The cells are harvested, resuspended in lysis buffer (50 mM Tris pH 7.5, 20 mM NaCl, 1mM EDTA, 1% SDS) and the soluble protein collected. This material is applied to 10-20 % gradient denaturing SDS PAGE. The separated proteins are transferred to nitrocellulose and the western blot conducted as above using the antibody reagents described. In parallel, the control oligonucleotides are added to identical cultures and experimental operations are repeated. Decrease in Beer mRNA or protein levels are considered significant if the treatment with the antisense oligonucleotide results in a 50% change in either instance compared to the control scrambled oligonucleotide. This methodology enables selective gene inactivation and subsequent phenotype characterization of the mineralized nodules in the tissue culture model.

SEQUENCES

Sequence ID No. 1: Human BEER cDNA (complete coding region plus 5 and 3' UTRs)

5

AGA FUNTOT GOTAUT GGAAGGTGGCGTGCCCTUTGGCTGGTACCATGCAGCTGCCANTGGCGCTGTGTGTGTGTGTGT OT MOTOSTA DACADAGOOTTOOGTGTAGTGGAAGGGCCAGGGGTGGCAGGCGTTCAAGAAT BATGCCAOGGAAAT DATCCC CGAGCT DGGAGAGTACCCCGAGCCTDCACCGGAGCTGGAGAACAACAACAACATGAACCGGGGAGAGAACGAGGGCGAC CTCCCCACCACCCTTTGAGACCARAGACGTGTCCGACTACAGCTGCCGGGAGCTGCACTTCACCGGCTATCTGACCGAT 10 AGETRET PROTECTES TEACHER TEACHER CONTRACTED TO THE TEACHER CONTRACT CANDED TO THE CALL TO THE TEACHER CONTRACT CANDED TO THE CALL TO THE CBCTTCCACAACCAGTCGGAGCTCAAGGACTTCGGGACCGAAGGCGCTCGGCCGCAAAAGCGCGCGAAAGCCGCGGGACCCCC CRESCRIBALROSCIRALGOCAACCAGGOGAGCTGGAGAACGCCTACTAGARGCCGGCGGGGGCGCTCCCCACCRGGGGGC SOCCOGGO TRANSCOGGOCGACATTTCTGTCCTCTGCGCGTGGTTTGATTGTTATATTTCATTGTAAATGCCTGC AGGGSTCCCACGGGGCAGGGGAATTGAGAGTCACAGACACTGAGCCAGGCAGCCCGCCTCTGGGGCCCCCCCTACCT TTGCT3GTCCCACTTCAGAGGAGGAGAAATGGAAGCATTTTCACCGCCCTGGGGTTTTAAGGGAGCGGTGTGGGAGTGG 20 CCRGA GCACARGACTGGGGGGCAACTGTAGATGTGGTTTCTAGTCCTGGCTCTGCCACTAACTTGCTGTAACCTTGAAC TRURCHATTUTOUTTOGGGACCTCARTTTOCACTTTGTARARTGAGGGTGGAGGTGGGAATAGGATCTUGAGGAGACTAT CASTISCATISATICASISCCARSSICACTICCASAATICASASITSISATSCICITITTSIACASCCAAASAISAAAAA CARACAGARARARAGAAAAGAGTOTATTTATGGCTGACATATTTACGGCTGACAAACTCCTGGAAGAAGCTATGCTG 25 CTTCCCAGCCTGGCTTCCCCGGGATGTTTGGCTACCTCCACCCCTCCATCTCAAAGAAATAACATCATCCATTGGGGTAGA ARAGGRGAGGGTCCGAGGGTGGGGAGGGRTAGARRTCRCRTCCGCCCARCTTCCCARRAGGAGCAGCATCCCTCCCCCG AGCCATCACAAACTCACAGACCAGCCATCCCTTTTGAGACACCGCCTTCTGCCCACCACTCACGGACACATTTCTGCCT AGRARACAGOTTOTTACTGCTOTTACATGTGATGGCATATCTTACACTARAGARTATTATTGGGGGARARACTACARGT GCTGTACATATGCTGAGAAACTGCAGAGCATAATAGCTGCCACCCAAAAATCTTTTTGAAAATCATTTCCAGACAACCAC TTACTTTCTGTGTAGTTTTAATTGTTAAAAAAAAAAAGTTTTAAACAGAAGACATGACATATGAAAAGCAGAGACT GGTCGTTTTTTTGGCAATTCTTCCACGTGGGACTTGTCCACAAGAATGAAAGTAGTGGTTTTTAAAGAGTTAAGTTACAT ATTTATTTTCTCACTTAAGTTATTTATGCAAAGTTTTTCTTGTAGARATGACAATGTTAATATTGCTTTATGAATTAA CASTOTGTTCTTCCAGAGTCCAGAGACATTGTTAATAAAGACAATGAATCATGACCGAAAG

35

30

Sequence ID No. 2: Human BEER protein (complete sequence)

MQLFLALCLVOLLVHTAFRIVEGQGVQAFKKTATEISFELGEVEEFFFELEKKITTMKAEKGGREHHEFETYSV.SE.37 FELHFTFYVTDGESFSAKFVTELVOSGQSSFARLLEMAIGEGNOVEFGSSESFROIEDPVFAQRVQLLSGGSGAFREFNIV

5 LVASCKCKRLTRFHNQSELKDFGTEAARPQKGRKPRPRARSAKANQAELENAY

Sequence ID No. 3: Human Beer cDNA containing Sclerosteosis nonsense mutation

AGAGOCTGTGUTACTGGAAAGGTGGCGTGUUCTCCTCTGGCTGGTACOLTGUAGCTGCCACTGGCCACTGGCCTGTGTTTGTCT OGRGOT DGGRAGATA DDDGGRGOCTG DRODGGRGCTGGRGRROLL DRAGA DDRT YAA DDGGGGGGRALLIGGRGGGCKKK OTOGOGRAGOSTTTTGAGAGGARAGAGGTGTGSGAGTAGAGGTGCGGGGAGGTGGAGTTGAGGGGGTAGGTGASSGAT 15 ABOTBOTGTGTGCCGGTGGTGAGGCGCGGGGGGGGGGGGGGAGAGGTGGTGGTGGTGGAAGTGGAAGGCCGGGGAAGGGCGCGAAGG CGCTTCCACARACAGICGGAGCTCAASGACTTCGGGAACCCGCGCGCAGAAGGGCCGGAAAGCGCCGGAGAGCCCCCG COCCOGRAGOGCARRACCARGOCRAGOCGAGATGGRARACGCCTRUTAGRAGOCGGGGGGGGGGCTCCCCRCCGGGGGGG 20 GCCCCGGCCCTGAACCCCGCGCCCCACATTTCTGTCCTCTGCGCGTGGTTTSATTGTTTATATTTCATTGTAAATGCCTGC AACCCAGGCAGGGGGCTGAGACCTTCCAGGCCTGAGGAATCCCGGGGCAAAGGCCCCCCTCAGCCGGCAGGACA TTGCTGGTCCCACTTCAGAGGAGGCAGAAATGGAAGCATTTTCACCGCCCTGGGGTTTTAAGGGAGCGGTGTGGGAGCAGTAT GARASTUCAGGGACT BETTARGARAGTTGGATARGATTCCCCCTTGCACUTGGCTGCCCATUAGRARGCCTGAGGCGTGC 25 CORGREDACARGACTGGGGGGAACTGTAGATGTGGTTTCTAGTCCTGGCTCTGCCACTAACTTXCTGTGTAAACCTTGAAAC TAGACARTTOTOCTTOUGGACCTCARTTTOCACTTTGTAARATGASGGTGGAGGGAGATAGGATCTCGAGGAGACTAT CASTIGOATIGATICAGIGCCARGGICACTICCAGARITICAGAGITGICATGCICTCTCTGACAGCCARAGATGRARAL CARACAGARRARALALAGAGTALAGAGTOTATTTATGGOTGACATATTTADGGCTGACARACTCCTGGARGAAGCTATGCTG 30 CTTCCCAGCCTGGCTTCCCCGGGATGTTTGGCTACCTCCACCCCTCCATCTCAAAGAAAAAACATCATCCATTGGGGTAGA AGCCATCACAAACTCACAGACCACACACCCTTTTGAGACACCGCCTTTCTGCCCACCACCACCACCACGACACACTTTCTGCCT 35 AGRARACAGCTTCTTACTGCTCTTACATGTGATGGCATATCTTACPCTARARGARTATTATTGGGGGARARACTACAAGT GCTGTACATATGCTGAGAAACTGCAGAGCATAATAGCTGCCACCCALALATCTTTTTGALLATCATTTCCAGACAACCTC

TER TOTTOT STOTE WITTITE ATT STEELE LEELE LEER STITTE AR DE SAR SURTET VAN TET VAR LEGOT WAS SANT G STANTITTT TOGGLAATT TIT COACGEGG A OTT STUDA CAR MAATGAAR STARTGOT TOTT AAAGA STIAAGTTA DRI ATTTATTT DI DACTTARSTTATTT RIGGEARA GETT TIT DIT STA MAGARTGACAATGI TARTATY SOTT TATGARTTAN DAGT DISTITCT TOGAGRG TOCAGRGA DATT STIARTARS SARAT SARTCRIGGARAG

5

Sequence ID No. 4: Truncated Human Beer protein from Sclerosteosis

MQUPLAUDLY DELYHTAFRYVEG*

10

20

25

30

Sequence ID No. 5: Human BEER cDNA encoding protein variant (V10I)

ABA SOCTGT GOTACTGGAAGGTGGCGTGCCCTCCTCTGGCTGGTACCATGCAGCTCCCACTGGCCCTGTGTCTCA . CTGCTGGTACACAGACTTCCGTGTAGTGGAGGGCDAGGGTGGCAGGGCTTCARGAATGATGCDACGGAAATCATCCG CGAGCTOGGAGAGTACCCCGAGCCTCCACCGGAGCTGGAGAGACAACACCCATGAACCGGGCGGAGAAAAAGAGGGGGGC CTCCCCACCCCTTTGAGACCAAAAAGTGTCCGAGTACAGCTGCGGGAAGCTGCACTTCACCCGCTACGTGACCGAT GRECONTROCRONAGESCONNICACESTERCESAGETSTRETETCOGGESCHATGEGGGGGGGGGGGGGGGGGTGCTGCCCARSS ABOTECTBTGT DOOGGTGGTGAGGCGCGCGCGCGCGCGAAGGTGCGCTGGTGCAAGTGCAAGTGCAAGCGCCTCACC COSTICOACAACCASTCGGAGCTCAAGGACTTCGGGACCCGAGGCCGGCAGAAGAGCCGGAAAAGGCCGGAAAAGCCGGGGCCCCG GCCCCGGCCCTGAACCCGCGCCCACATTTCTGTCCTCTGCGCGTGGTTTGATTGTTATATTTCATTGTAAATGCCTGC AGGGGTCCCACGGGGGAGGGGAATTGAGAGTCACAGACACTGAGCCACGCAGGCGCGCTCTGGGGCCGCCTACCT TTGCTGGTCCCACTTCAGAGGAGGCAGARATGGRAGCATTTTCACCGCCCTGGGGTTTTAAGGGAGCGGTGTGGGAGTGG GRANGTCCAGGGRCTGGTTARGARASTTGGRTARGRTTCCCCCTTGCRCCTCGCTGCCCATCAGRRAGCCTGAGGCGTGC CCAGAGCACAAGACTGGGGGCAACTGTAGATGTGGTTTCTAGTCCTGGCTCTGCCACTAACTTGCTGTGTAACCTTGAAC TACACAATTCTTCTTCGGGACCTCAATTTCCACTTTGTAAAATGAGGGTGGAAGGTGGAATAGGATCTCGAGGAGACTAT CAGTTGCATTGATTCAGTGCCAAGGTCACTTCCAGAATTCAGAGTTGTGATGCTCTCTTCTGACAGCCAAAGATGAAAAA CANACAGANALANAGTANAGAGTCTATTTATGGCTGACATATTTACGGCTGACAAACTCCTGGAAGAAGCTATGCTG

ASSOCRT TH DRAW TO CACAGA TO A STACKT OF UTITITIES ASSAURT TO A STACKT THE USUAL TACTOR AND A STACKT TO THE CONTROL OF THE CACAGA CACAGA TO TO THE CONTROL OF THE CACAGA C

(V101) Sequence ID No. 6: Human BEER protein variant

MQLF LALGLICLEVHTAFRVVEGQGMQAFMMDATEITRELGEVERES SELEMMETMMRAENGGF SEHAFFETHDVGEVSC FELHFTAVVTDGS CRSAMEVTELVOSGQCGFARLLEMAIGRGMMWREGGSDFROIS DRYRAQRVQLLOFGGERSRASHVB LVAGOMUNALTREHMQGELMDFGTEAARFQNGRMFRSRARGAMAMQAELEMAY

15

Sequence ID No. 7: Human Beer cDNA encoding protein variant (P38R)

ASAGOTT STGGTACTS SAAGGTGGCGTGCCTCTGGCTGGCTGGTACCATGCAGCTCCCACTGGCCCTGTGTCTCGTCTGT 20 CTGCT30TACACACACCCTTCCGTGTAGT3GAGGGCCAGGGGTGGCAGGGGTTCAAGAATGATGCCACGGAAATCATCC $oldsymbol{G}$ CBAGCTCBGAGAGTACCCCGAGCCTCCACCGGAGCTGGAGAACAACAACAAGACCATGAACCGGGGGGAGAACGGAGGGCGGC OTGCCCACCACCCTTTSAGACCARRGACGTGTCCGAGTACAGCTGCCGCGAGCTGCACCTCACCCGCTACGTGACCGAT SAGCCATG DU GENECISCORRAGOGGTER DEGRACT GET BTGCT DU GAUTRAGGGCCGGGGGGGGGGTGCTGCCGRACGC 25 ABSTROTETETCOSGETGAGGCGCCCCCCCCCCAAGGTGCGCCTGGTGCCAAGTGCAAGTGCAAGGGCCTCACC GCCCCGGCCCTGRACCCCGCGCCCACACTTCTCTCCCCCTCTGCGCGTGGTTTCATTGTTATATTTCATTGTARATGCCTGC AACCCAGGGCAGGCCGAGACCTTCCAGGCCCTAAGGAATCCCGGGGCCGAGGACAAGGCCCCTCAGCCGGCCAGCCGAGACAAGGCCGAGCAGAGCTG AGGGTCCCACGGGGCAGGGGAATTGAGAGTCACAGACACTGAGCCAGCAGCCGGCCTCTGGGGGCGGCTACCT TTSCTGGTCCCACTTCAGAGGAGGCAGAAATGGAAGCATTTTCACCGCCCTGGGGTTTTAAGGGAGCGGTGTGGGAGTGG GARASTOCAGGGACTGGTTAAGAAAGTTGGATAAGATTCCCCCTTGCACCTGGCTGCCGATCAGAAAAGTCTGAGGCGTGC COAGRECACRAGROTGGGGGURROTGTRGRTGTGGTTCTAGTUCTGGCTCTGCCRCTRROTTGCTGTGTRAGCTTGRRC TACACAATTCTCCTTCGGGACCTCAATTTCCACTTTGTAAAATGAGGTGGAGGTGGAATAGGATCTCGAGGAGACTAT

15 Sequence ID No. 8: Human Beer protein variant (P38R)

MQLELALCLVCLLVHTAFRVVEGQGWQAFKNDATEIIRELGEYEEFFEELENNKTMNRAENGGREEHHEFETHDVGEYSC RELHFTRYVTDGECRSAKEVTELVGSGQCGEARLLENAIGRGFWWRESGEDFRCIEDRYRAQRVQLLGEGGEAFRARKVR LVASCKCFRLTREHNQSELKDEGTEAAREQKGRFFRERARSAKANOAELENAY

20

Sequence ID No. 9: Vervet BEER cDNA (complete coding region)

35

Sequence ID No. 10: Vervet BEER protein (complete sequence)

NQUE LA LOUVOLLYHAR FRYYERQRWQA FRODATEI DE LIFEY FER EELEMBYTKOK AEMBE EHHEFET HOVBEY FO RELHFTRYVTEUR ORBAKRYTELY DROOGFARLLEMAD RARRWRES DE DFROIE DRYRAQRVQUL DE KRAREAS FUK LVARONOFRLTREHNQSELKEFGRERAR FONGREER FRAR RAKRNQRELEMAY

5

Sequence ID No. 11: Mouse BEER cDNA (coding region only)

APPORTUGAÇÃO DE CARA SECUCION DE TOTO DE CONTRETE DE CARACTE DO TROTA DE SUBSTITUTO DE CONTRETE DE CON

20 Sequence ID No. 12: Mouse BEER protein (complete sequence)

MQFSLAFCLICLLVHAAFCAVEGQGWQAFRNDATEVIFGLGEVFEFFFERHQTMNRAEHGGRPFHHFYDAHDVSEYSCRE LHYTRFLTBGFCRSAHFYTELVCSGQCGFARLLFHAIGRVFWWRFHGFDFRCIFDRYRAQAYQLLCFGGAAFRSRKVRLY ASCHCHRLTRFHHQSELKDFGFETARFQKGRKFRFGARGAKAHQAELEHAY

25

Sequence ID No. 13: Rat BEER cDNA (complete coding region plus 5' UTR)

TO SAR BOTTAR BRATTO WAR DUT WARROUS DE BORBOOR CARRAR BOTT IN JAAR SU AUREN DO BOTTO SERVAR BOTTA ABOURA DOAR BORBARUT BORBAR OBOOTA OT AB

5 Sequence ID No. 14: Rat BEER protein (complete sequence)

MQUSUAFOLACULVHRAFYAVESQSMQAFRHDATEIIFGLREYFEFFQELENHQTKHFAENGSRFFHHFYDTFIVSEY. 1 RELHYTFFVTDSFCRSRFFVTELVOSQQCGFARLLFHRIGRVKWWKFHGFDFRCIFCFYFAQRVQLLGFGRAAFFSFFJR LVASUN MFLTEFHIQSELFDFGFETRRFQFGRFFFFRRARGAMMQAELENAY

10

Sequence ID No. 15: Bovine BEER cDNA (partial coding sequence)

- Sequence ID No. 16: Bovine BEER protein (partial sequence -- missing signal sequence and last 6 residues)

NDATELIFELGEYFEFLFELNIKTKURAENGGREFHHEFETKDASEYSCRELHFTRYVTDGFCRSAKFVTELVUSGQOGF ARLLPHAIGRGKWWRESGEDFROIFDRYRAGRYQLLOFGGAAFRARKVRLVASCHOKRLTRFHNQSELFDFGFEAARFQT GRELRFRARGTYAGRA

30

Sequence ID No. 17: MluI - AviII restriction fragment used to make mouse Beer transgene

35

CGCGTTTTGGTGAGCAGCAATATTGCFCTTCGATGAGCCTTGGCGTTGAGATTGATACCTCTGCTGCACAAAAGGCAATC

WO 00/32773

VACCINATION OF TRACTAGOR CATTOSTRACATOR TO TO TOTAR CATTOR OF A TARGOR STATIONAL PRABAR CHOOSE TRATORCARAT BOTGOTATOCA DO CARGO CARACO CARAR CONTOS SO CONTORATATOTA CARACATO CARGO CONTOS C ATOUT SOFT GAT GAGUDAGOGGRGAR GARGETTARCOGT CRETGOUGATARSTTURRASTTARACUT GGT GTT GATACORR CATTGRARAGGTTGRARAROGCGCTGRARARAGGCTGCTGRATGTGCGGCGCTGCTGGAATGTCRCRARAGCRARAGGRARAGGRA ACARGRAR DOGREGATGRAFGEGETTEGTAT PTUGGCREGGEGATCAT PRTGGRATGTTT DOGGGTGGTGTTATET PC CAGCART SPORT SCATARTACCARTTGATAACTACTATCATTTGGGGRETCCTTTDGGGGGGATCGGCCTTRETAGGGGRE GGCGACCTGGCGGGTTTTGGCTATTTATGALLATTTTCGGGTTTLAGGCGTTTLAGGTTTCGGGTCTTCCTTCGTGGTALLCTTALTGT TO BOGGOOGOATAATAO GACTOROTATAGGGATOGAO BOOTAO DO COO SORTGAABO GAGGAGOTGGA OTOC BOATG COCAGAGAGGCCCCCAAACCCCCCAAAGTGCCTGACCTCAGCCTCTACCAGCTTGGCCTGGGCTTGGGCCGGGGTCAAGGC TACCACATT CTCTTAACAGGT GGCTGGGCTGTCTCTT GGCCGCGCGT CATGTGACAGCTGCCTACTTCTGCAGTGAGGTC RECOTOGRATGY OTTOGTTGCCATGGCARCGGGATGACGTTACARTCTGGGGAGCTTTTCCTGTUCGTGTCA GGARAT DURERTROCOTARARTROCOTAGRAGRAGRAGRAGITAGOTGAGODAR SSOTTTOOT GGOTTOTOGAGRATARAGTTTG ACTTAGATGGAAAAAACAAAAGATGATAAAGACCCAGCGAGCCATCTGAAAATTCCTAATTGCACCACTAGGAAAATGTGTA 15 TATTATTSASCTOGTATGTGTTCTTATTTTALALAGALLACTTTAGTCATSTTATTALALALATTTCTCAGCAGTGGGA GAGAACCARTATTARCACCAAGATAAAAGTTGGCATGATCCACATTGCAGGRAGATCCACGTTGGGTTTTCATGAATGTC AAGRECECRTTTATTARAGTECTARGETETTTTTTGCACRETAGGRAGGGGATGGCTGAGGGGGTTATAAGG ATCTTTCAATGTCTTACATGTGTGTTTCCTGTCCTGCACCTAGGACCTGCTGCCTAGCCTGCAGCAGAGCCAGAGCGGGTT TCACATGATTAGTCTCAGACACTTGGGGGGCAGGTTGCATGTACTGCATCGCATCCCATACGGAGCACCTACTATGTG 20 TCRARCACORTATGCTGTTCACTCTTCAGARCGGTGGTGGTCATCATGCTGCATTGCTGGCTGGATGGTTGGATGGTAGAT GAGCTGAGATATATGGACGCACTCTTCAGCATTCTGTCAACGTGGCTGTGCATTCTTGCTCCTGAGCAAGTGGCTAAACA AGARGOCAGGGGGCTTGGCGGCTCTCAGGAGCCTGCTTGCTGGGGGACAGGTTGTTGAGTTTTATCTGCAGTAGGTTGCCT AGGCATAGTGTCAGGACTGATGGCTGCCTT3GAGAACACATCCTTTGCCCTCTATGCAAATCTGACCTTGACATGGGGGGC 25 GCTGCTCAGCTGGGAGGATCAACTGCATACCTAAAGCCAAGCCTAAAGTTCTTCGTCGACCTGAAAACTGCTGGACCAAG GGSCTTCCGGGCACATCCTCTCAGGCCAGTGAGGAGTCTGTGTGAGCTGCACTTTCCAATCTCAGGGCGTGAGAGGCAGA GGGAGGTGGGGGAGAGCCTTGCAGCTCTTTCCTCCCATCTGGACAGCGCTCTGGCTCAGCAGCCGATATGAGCACAGGC 30 TATCCTCTCTTAGGTAGACAGGACTCTGCAGGAGACACTGCTTTGTAAGATACTGCAGTTTAAATTTGGATGTTGTGAGG GGARAGGGARGGGCTCTTTGACCATTCRGTCARGGTACCTTCTRACTCCCRTCGTATTGGGGGGCTACTCTAGTGCTRG ACATTGCAGAGAGCCTCAGAACTGTAGTTACCAGTGTGGTAGGATTGATCCTTCAGGGAGCCTGACATGTGACAGTTCCA CAAABAACTGACAGACGGAAGCCTTGGAATATAAACACCAAAGCATCAGGCTCTGCCAACAGAACAGTCTTTAACACTCA 35 GGCACAGGACGATATAAGTGGCTTGCTTAAGCTTGTCTGCATGGTAAATGGCAGGGCTGGATTGAGACCCAGACATTCCA

TOOTCACAACATAGGAATTGTGATAGCAGCACACACCCCGGAAGGAGCTCGGGAAAATCCCCACAGAGGATTCCGCAGAGAATG ACAGGOGRATGOCTACRORGRAGGTGGGGRAGGGRAGGRAGGRAGGRACKSTATGGGCGTGGGCGTGGGRACKAGTCTRTTTGGGG AAGUTGOOGSTAA.COGTATATGSCTGGGGTGAGGGGAAGAGTCATGAGATGAGGCAYUAAGAGCAGAGCAGGCAGGCAG TACGGGCTGCTTATTGCCAAGAGGCTGGGATCTTCCTCCTCTTCCTCCTCCTCCTGGGGCTGCCTGTTCATTTTCCACGACC OTTOURATION STORESTONES AND ACCORDING TO SUBJECT TO SERVED THE STOREST OF SANTONES AND SERVED TO SERVED TO SERVED THE SER FCARGTOCTTCATSCACGTCAACACCAGCTAGCTTCTACGAGGATTTTTGCATCACCTACTTGGGTATCAAGGCCAAT GATE USCASSASTICARCOTCAST SCTTACTITGRAAGGGCCRCAGATTT CATTGACCAGGGCTGGGCCCATRARATTC TARRIBAR DITACATTRUGUCACICAT REAGOSTAR SOCOTOT SUGACOTGOTTUOT CUARRIA RECOLUCACTOGRARAR 10 SSTTOCASAARSATCOCAARATATGOCACCARCTAGGGATTARGTGTCCTACATGTGAGUCGATGGGGGCCACTGCATAT ASTOTOTOCCATAGACATGACAATGGATAATAATATTTCAGACAGAGACAGAGAGATTAGGTAGCTGTUUTCCCTT TAATTSAGTSTUGGCATTTTTTTTTTCATGTATGTGTATACATGTGTGTGCGCACACACGCATAGGTTCATACTGAACACC STOTTURATUSTTUUGURGUGGRUUTTATTTTTTGRGGGRGGTCTOTTUUUTGRTUUTGGGGGTCATTGGTTTRTTTRT POT SUTGGUCASTGASCTCTGGASTTUTGCTTTTCTCTAACCTCCCTA SUUCTGGGACTGCAGGGSCATGTGCTGGGCCAG 15 GOTTTTATGT0G0GTTGGGGRTCTGRACTTAGGT000TAGG00TGAG0RUGTARAGRUTUTG00R0ATG100CAG00TGT TTGAGCAAGTGAACCATTCCCCCAGAATTCCCCCCAGTGGGGCTTTCCTACCCCTTTTATTGGCTAGGCATTCATGAGTGGTC ACCTOGCCAGAGGARTGAGTGGCCACGACTGGCTCAGGGTCAGCCTAGAGATACTGGGTTAAGTCTTCCTGCCGCTC ACAGGSCAGATGTCAGCAGAGCAGACAGGTTCTCCCTCTGTGGGGGAGGSGTGGCUCACTGCAGGTGTAATTGGCCTTCT 20 OTTACAGAAGTTCTATTGACTGGTGTAACGGTTCAACAGCTTTGGCTCTTGGTGTAGGGCGGTGCATACTGCTGTATCAGCTC GSTORGTGROTGGGCRTTTCTGRRCATCOCTGRRGTTAGCRCRCTTTCOUTCTGGTGTTCCTGGCTTARSROCTTCTAR ATCTATATTTTATCTTTGCTGCCCTGTTACCTTCTGAGAAGCCCCTAGGGCCACTTCCCTTGCACCTACATTGCTGGAT 25 GGTTTCTCTCCTGCAGCTCTTAAATCTGATCCCTCTGCCTCTGAGCCATGGGAACAGCCCAATAACTGAGTTAGACATAA ARAGGTOTOTAGCOARAROTTOAGCTARATTTAGROARTARATOTTROTGGTTGTGGARTCCTTARGATTCTTCATGROO ACCTSCTCAAGGAAGGAACAAATTCATCCTTAACTGATCTGTGCACCTTGCACAATCCATACGAATATCTTAAGAGTAC 30 TAAGATTTTGGTTGTGAGAGTCACATGTTACAGAATGTACAGCTTTGACAAGGTGCATCCTTGGGATGCCGAAGTGACCT SCTETTCCAGCCCCCTACCTTCTGAGGCTGTTTTGGAAGCAATGCTCTGGAAGCAACTTTAGGAGGTAGGATGCTGGAAC AGCGGGTCACTTCAGCATCCCGATGACGAATCCCGTCAAAGCTGTACATTCTGTAACAGACTGGGAAAGCTGCAGACTTT ARGGCCAGGGCCTATGSTCCCTCTTARTCCCTGTCACACCCRACCGGAGCCTTCTCCTCCAGCCGTTCTGTGCTTCTC CCTCATTCAGGGAACTCTGGGGCATTCTGCCTTTACTTCCTCTTTTTGGACTACAGGGAATATATGCTGACTTGTTTTGA CCTTGTGTATGGGGAGACTGGATCTTTGGTCTGGAATGTTTCCTGCTAGTTTTTCCCCCATCCTTTGGCAAACCCTATCTA

WO 00/32773

TAT DITA MEDIAGRATARI REDD DI DESTIDI REARDITE MEDIARRIT RETITORE REMINAT PARI DE PROPERTI MEDIA COCCERNETAT BYTSTTDACAGRITATTÖA TEVESAGTBETTBUTVAR DAAR VEGESAGRITA ARTU MAVA UNT SVARTACK. THE TIGGET AND TRANSPARANCE AND THE CONTROL OF THE CARD SECTION OF THE CONTROL OF THE CONTROL OF THE CARD SECTION OF THE CARD TROTHARRIERAT REATAUTRRUTTET OTAT ONA OTT ROARGRAUT OTAGRREA. NA RRAAT OVATTA IT BARAAAA. V GCAARCTGCCTRRCCRTARCRARARCRATGRCCTGRCCTCTCCCCTGTGRGAGATCCTCCCCTTRACATATARCRAGAGAA TRARA CATTARA GRAVACAGTAGAT GOCARTTTTARGOUDO DAVATGOA VATORADAR ATGOGRATTT VARVACACATAT GCACTCATGT BRACCADGCATGCACACTC333CTTATCACACACATARTTTGARR BAGAGAGTGRAFAGACACACACACAC ATTAGASTTOACASSARASTSTGAGTGASCACACCCATSCACAGACATSTSTGCCAGAGAGAGAGAGAGAGAAGSAGCCTGS/ 10 TTTGTGTATARARAGARGCATCATGTGTTTCTARGEAGGGGTGTGARGGAGGGGTTGTGTGGGACTGGGAACTGGAACAT GOTTGTARCTGRGCRTGCTCCCTGTGGGRARACRGGRGGGTGGGCCRCGGGGGAGRGGGTCCCRTGTCCRGCGGGGGGAATCRGT ARARGUDOCT SCT GAGRACTT DA GGTARTAG DORGAGAGARAGARAGGTA GGARAGT 9606 GGACT OCUATOT UT GAT GTAG GAGGATCTGGGCRAGTAGAGGTGCFTTTGAGGTAGAAAAGGGGTGCRGAGGAGAGATGCTGTAATTCTGGGCUAGCAGTT TOTTTOCARATARTSCOTGTGAGGAGGTGTAGGTGGTGGCCATTCACTCACT TRACAGGGGATGATGATGCCCCGGTGGA 15 TGCTGGF.FAT SGCCGAGCATCAR OCCTGGCTCTGGAAGAACTCCATCTTTCAGFAGAGAGAGTGGATCTGTGTATGTAT CGGGGTCACAGGTGCTTGGGGGCCCCTGGGGGGACTCCTAGCACTGGGTGATGTTTATCGAGTGCTCTTTTGTGCCAGGCAC AGCTAGOGGCARGGGTAGAGGGGGAGCTOCCTGTGCAGGAGAGATGCARGCAAGAGATGGCAAGCCAGCCAGTGAGTTAAGCAT GRAGGAGGGCRGCTCCCTTGCAGTTTTARAGATTTTTTCCTGACAGTGRCCTTTBSCCTCTCCCTCCCCAACTTCCCTTCCT TESTTTSTT CCCASCATTGCTTTSCTTTSTSSTTGASARRTTSTGAGTTTSCASTTSACTGGTSATGCAGAGSGARAGAG 25 GTATGTSTOTCASTGGGAATGGCTCATAGTCTGCAGGAAGGCAGGGAAGGAATAAGCTGTAGGCTGAGGCAGTGTGG GATGCAUGGAGAGAGAGGAGGAATACCAGAGAAGAAGTAATTAAGGGAGCTACAAGAGGGCATTGTTGUGGTTGTGTGTG GTGTGTGCCTGCATGAGTTCATGTGTGCCACGTGTGTGCGGGGAACCCTTGGAGGCCACAAGGGGGCATCTGATCCCCTGG AACTGGAGTTGGAGGAGGTTGTGAGTCCCCTGACATGTTTGGTGGGAACTGAACCCCGGTCCTATGCAAGAGCAGGAAGT 30 GCAGTTATCTGCTGAGCCATCTCTCCAGTCCTGAAATCCATTCTCTTAAAATACACCTGGCAGAGACATCATGGGATTTA CGTATGGATTTAATGT9GCGGTCATTAASTTCCGGCCACAGGCAAGCACCTSTAAAGCCATCACGACAACCGCAACAGT3A ATGTGACCATCACCCCCATGTTCTTCATGTCCCCTGTCCCCTCCATCCCCATTCTCAAGCACCTCTTGCTCTGCCTCTG 35 GTGTATGCACATGTGCACATGTGTACAGATACTATGGAGGCCAGAAGAGGCCATGGCGTCCCTGGAGCTGGAGTTACA

GRICK FOTOT DE ROTRO DE ARTRE SERTIR ET RRICKE L'ERRECTE DA TIMER PORRECRITOTERROT DITTER ROMA P TOTOR STAULATTOST CARTANT NOWNER SWITCHAIT STATEN, 18 NAT STARWITA SAATATOTT VATTAT STARTS TGTACATTETTTTGT SOTAAGAGA GASTAAT SOTGTATAGGGT SABOT GGGGTGAAGGTTGGGGATGGTGGTGGGGTUAGG TO STICHT STORAGT GOTAGGAT GAGAGGGGAGT GOTAACTTAGAT GGT TO JAT STOTT GTT CAAGAGT GAAGGGAT AF HAT TURTROAGRARAGIOTSS STORCHARGIST SORGIT CROT SARTSSOR CARGO COST SATURR SARROARRACIORAS : -OT GRADART PROACT GAIDTOT TOT TO CARAGET DORGE STORART COURRINA MORGAT GROSS TO A CARGOTAT MT ACCACT STITCA ACCACACACACACACT STITCA COTTOTICA COTTOTICA COTTOTICA CACACACACACT STITCA COCCCCC 10 CORTGORIGGORGRAFTSTORGGORIGTORGROTATTSTGRADAGOGGROAGTTOTTTTTTTCRATCATSTGRATTDDRA APRIT GRAST CARRETTAT GT GT GT GGCAG CARACT SOST TO ACCURST GRAGA CAT CT CORTATT CT T T T T T T T COC ST C AGGTGGGGGGTTGTTGGATAGGGGAAAGTGGGTTTGGAGTTGGAGTTGAAAGTGAGTGAGTGTGTGTGTGAGAGTA TTGGARTTAGGATGTGTAUTAGGRGAGGTGAGTGATTARTTUTTTGATGGGGGGGAAGGGGAGATGGTGGAGG TGRAG PRATGACTGGACTGGACATGAGCSTG RAAGCCAGAGAACARCTTCAGTCTAATGCTCTCCCAACTGAGCTATTTT GGTTTGCCAGAGAACAACTTACAGAAAGTTCTCAGTGCCATGTGGATTCGGGGGTTGGAGTTCAACTCATCAGCTTGACAT TGGUTTTTTACCCACTGAGCCTTCTCACTAUTCTCTACCTAGATCATTAATTUTTTTTAAAAAGACTTATTAGGGGGC TGGRGRGATGGCTCRGCCGTTRRGAGCRCCGRATGCCCTTCCRGAGGTCCTGRGTTCRRTTCCCAGCATGCCATTGCTGG CCTGGTCATTCTGGGTGGGTGCATGGTGATATGCTTGTTGTATGGAAGACTTTGACTGTTACAGTGAAGTTGGGCTTCCA 20 CASTILACCACRITCTCCCCCTGTTTCTTGCAGGCGGGGGGGTGCTTGTCCATTGCCGCGAGGGGAGGGTACAGCCGCTCCCCCAACGCTA GTTATESCETACET SATGATGEGGEAGARGATGGACGTCARGTCTSCTCTGAGTASTGTGAGGCAGAATCGTGAGATCGS CCCCAACGATGGCTTCCTG3CCAACTCTG3CCAGCTCAATGACAGACTA3CCAAGGAGGAGGGAAAGGTGAAACTCTAGGGTG CUCRCAGCTTTTTTGCAGAGGTCTGACTGGGAGGGCCCTGGCAGCTTTTAGGAAACACAGTATACCCACTCCCTGC ACCACCAGACACGTGCCCACATCTGTCCCACTCTGGTCCTCGGGGGGCCACCCTTAGGGAGCACATGAAGAAGCTC 25 TCTSTGGCATAGATACATOTCAGTGACCCAGGGTGGGAGGGCTATCAGGGTGCATGGCCCGGGACACGGGCACTOTTCAT GACCCCTCCCCCACCTGGGTTCTTCCTGTGTGGTGTCAGAACCACGAGCCTGGTAAAGGAACTATGCAAACACAGGCCCTG ACCTCCCCATGTCTGTTCCTGGTCCTCACAGCCCGACACGCCCTGCTGAGGCAGACGAATGACATTAAGTTCTGAAGCAC AGTGGAGATAGATTAGTGACTAGATTTCCAARAAGAAGGAAAAAAAAGGCTGCATTTTARAATTATTTCCTTAGAATTAR AGATACTACATAGGGGCCCTTGGGTAAGCAAATCCATTTTTCCCAGAGGCTATCTTGATTCTTTGGAATGTTTAAAGTGT GCCTTGCCAGAGAGCTTACGATCTATATCTGCTGCTTCAGAGCCTTCCCTGAGGATGGCTCTGTTCCTTTGCTTGTTAGA AGRGCGRTGCCTTGGGCRGGTTTTCCCCCTTTTCRGRRTACRGGGTGTARRGTCCRGCCTRTTACRRACRARCRARCRAR GCCAAGTGCTTGCCATCCTGGTTGCTATTCTAAGAATRATTAGGAGGAGGAGGAACCTAGCCAATTGCAGCTCATGTCCGTGG GTGTGTGCACGGGTGCATATGTTGGAAGGGGTGCCTGTCCCCTTGGGGACAGAAGGAAAATGAAAGGCCCCTCTGCTCAC

WO 00/32773

DEFORMING TOTAL PARAGEDED ROT RATE TO TOT RETENDADE OF THE PARAGED TO THE AREA OF THE OTHER RALE FRANCE OF DEPTH AND THE RALE FRANCE OF THE RESERVOIR OF THE RALE FRANCE OF THE RALE FRA ACTTURIUM SAO SATRABARTURBARARARARARARARARARARARARARARARAR MOUTOTAAAA MAACTTT MIAAA MOID ROTTT T SARCOT COCTOGRECATOROGERATOROGERACITOS CLAGRICATO SARA PATRAS CUTOTE SAGRICTO CAL OTOCATORE TO STATIST OT COURGINATION CACAGAR COCATARACCA COLONO ACACATAR CONTRA CATALLA ACCOCATAT TRECTRACT STARGAT STETTACAGRASTTTGCARARRASARAR COTTOCTT BARGRAPATATCAGRARATTTTT STORA ADATTTCHERTTCHGCTTTAAGTGTAAGTCAGCAGTGTTCHTCHTTGTAAAGAAGTTCCCTTTTCCCAGAGCTGCCTTTTCCCAGAGCTGCC GURRARAGO PROTESTANDAGA COTATUTTA GARGATUR DI NOCAG NATRAR SA DOTOT NATURACA DE DAGARANA GARGOT N TGCAGCCTGWATGSTCATTGTCCCCTRTTCTGTSTSACURCAGUAR DOCTSGTCACATAGGGCTGGTCATCCTTTTTT TITTTTTTTTTTTTTTTTTGGCCCAGARTGAAGTGACDATAGCCAAGTTTTTCTTAGTTTAGTTTTAGTTTTGCAAG CTOTTGOTCRATACHATGTGCATTTCRARATARCROTGTAGRGTTGRCAGARCTGGTTCRTGTTRTCRAAAJAGGRARA GRGADGRANGERGERAGERARGRARAGRARAGRARAGGRARARGRI UTGGGUTADGRGGRAGGGRTGRTTGGRATGTGTAGRA GCCDASTTCATGAGAGGCAGAGACAGGAAGACCGCCGAAAAGGTCAAGGATAGCATGGTCTAGGTATGGAGACTCCAGCCA SGSCTACUSTOCCAAGATCCTAGSTTTTGGATTTTGGGCTTTGGTTTTTGAGALAGGGTTTCTGTGTGTAGCCCTGGCTG TOCTGARACTOGOTOTGTAGACCAGGCTGGCCTCARACTTAGAGATUTGCCTGACTCTGCCTTTGAGGGCTGGGACGRAT 15 CATGACTTT RAGOCATCTCCAGAGAAGGAAGTGAAAATTGTGGCTCCCCAATTGGGAACACAGTCTCTCTTTGTCTA GSTARCACATSGTGACACATAGCATTGAACTCTCCACTCTGAGGGTGGGTTTCCCCCCCTGCCTCTTCTGGGTTGGTC ACCCCATAGGACAGCACAGGACAGTCACTAGCACCTACTGGAARCUTCTTTGTGGGAACATGAAGAAAGAGCCTTTGGG 20 AGATT DOT GGOTT TO CATTAGGGOT GAARGTACAR OG STTOTT GGTT SGOTTT GGOT COTGTT TATARRACTAG OT ROTA TTOTTCAGGTARRATACOGREGATGTTGTGGRARAGCCARCOCCGTGGCTSCCCSTGAGTAGGGGGTGGGGAATCCTG COACTITICTAT GROTTATARACATOCAGGTARRATTACARACATARRATGGTTTCTCTTCTCAATCTTCTARAGTCTG CONGCCTTTTCCAGGGGTAGGTCTGTTTCTTTGCTGTTCTATTGTCTTTGAGAGCAGACTAACACTTACCAAATGAGGG 25 AACTETTGGCCCATACTAAGGCTCTTCTGGGCTCCAGCACTCTTAAGTTATTTTAAGAATTCTCACTTGGCCTTTAGCAC ACCCCCCACCCCAAGTGGGTGTGGATAATGCCATGGCCAGCAGGGGGGACTGTTGAGGCGGGTGCCTTTCCACCTTAAG TTGCTTATAGTATTTARGATGCTARATGTTTTARTCARGRGARGCACTGRTCTTRTARTROGRGGATARGAGATTTTCTC ACAGGRAATTGTCTTTTCATAATTCTTTTACAGGCTTTGTCCTGATCSTAGCATAGAGAGARATAGCTGGATATTTAACT TGTATTCCATTTCCCTCTGCCAGCGTTAGGTTAACTCCGTAAAAAGTGATTCAGTGGACCGAAGAGGCTCAGAGGGCAGG 30 GGATGGTGGGGTGAGGCAGAGCACTGTCACCTGCCAGGCATGGGAGGTCCTGCCATCCGGGAGGAAAAGCAAAGTTTAGC TSTTTCCTTTTGTGTGTTTGSGCTTTTTATGTGTGCTTTAT&ACTGCTGTGGTGGTGGTGTTGTTAGTTTTGAGGTAGGA ARRAGORCATGCCACCACACACACATACAGCATTTTTTTTAACATTTAARRATAATCACCTAGGGGCTGGAGAGAGAGGGTTCCA 35 GCTAAGAGTGCACACTGCTCTTGGGTAGGACCTGAGTTTRGTTCCCAGAACCTATACTGGGTGGCTCCAGGTCCAGAGGA

THUS ASSUMD OT ABOUT OURS ARE HEAD TO TO THAT IS THE TREE OF A PARTA OF THE TREE STARS OF AN EXPERIENCE AS A S OTTTERRECOTOUTARRECOTA VI USTTORRAGGA CONTORGARA SOTIO SORTA ITUTORRARATI VATOT VATIOT VATIOTI PARA TTUTGGCTAACGTAAGACTTACAWAGACAGAAAAGACTCAGGGTGTWTWWWGGTTGWGATGGAWAAGAGAAGATWAGT AGGGGGAGCACGGGGAACTTGGGCAGTGAAAATTCTTTTGCAGGACACTAGAGGAGAGATAAATACCAGTCATTGCACTCAC TACTUGA CAACT COAGGGAATTAT SOTGGGT GAAAAGAAGAAGGCCCAGGT ATT WACTGCATT SUCTGCATTT COGTAA ATTITTTTARATT MARRAGRARAR GATGTARAT CARBOTTAGATGA ST SOTT SOTTGAG STORGAGATGAGA SA PACATOTAPACAR OT COAT CARRAR DOWN CAGRARAGEA DOGGOT STRUT SACRA COTACTATATOT CONCOCCOSQUES STGAT DAAGGTTA GOODT CAGOTARD OT STGGT BOAT GA GATOOT STTTCARRAA OTTTRATARAGRAATRADGAARARA SACATCARRECAGATCOTTEGGS CCAPAGGCSGACAGGCGAGTCTVSTOSTAAAGGTCGTGTAKAAGCGGGATSCATGAGC TO CITE DATE ADECEMBET CATE BOTH TO LEGISLAND SATERATE SALTERATE DE LA RABADTAC TADES ADECEMBENTO DE LA CATE DA CATE D 10 TTORAGRACTOTOTATTACARTTATOTORARATATTARAFIRERROGARRATTARRARAGRARARACTATOCAGGTGTG GT GGT GT GUACCTATA GCCAUGGG CACTTU GAAAGCT GGAGCAAGAGGAT GGOGAGTTTU GAAGGTATUT GUGCCT GTACA GCARGACORTOCOCARACCARACCARACAGCARACUDATTATGTCACACARGAGTGTTTATAGTSAGCCCCCCC SAGA SCATGGGGTGGGGGTGGGGGGTGGGGGA CAGARATATUTRARCTSCAGTCAATAGGGATCCACTGAGACCCTGGGGC 15 TTSACTGCAGCTTAACCTTGGGARATSATAAGGGTTTTSTGTTGAGTAAAAGCATCGATTACTGACTTAACCTCAAATGA AGRARALGALERRAGGERRAGRAGERRAGOCARAGGGCTGGTGAGATGGCTCAGTGGGTARGAGGAGCGGACTGG TOTTCOGRAGOTCCAGAGTTCAARTCCCAGCRACCACTGGTGGCTCACAACCATCTGTAACGAGATATGATGCCCTGTT 20 COSAGGRARCORGGOOGGRARCESRRGSCRGGCRGGROGRGGGRGGRGGRGGRGGCRTCCTGTGRRRRGGRAGGCTRCC CATGGGCCGAGGAGGGTCCAGAGAGATAGGCTGGTAAGCTCAGTTTCTCTGTATACCCTTTTTCTTGTTGACACTACTTC AATTACAGATARRATARCARATARRORARATOTAGAGOOTGGCCROTCTGTGGCTGGCTTGATTTTTDCTGTTACGTCCAG CAGGTGGCGGAAGTGTTGCAAGGAGAGAGTGGCATTAAGGTGGCCAGCATAATCTGCCATCAGCAGGTGGTGCTGTGA GRACCATTAT90T9CTCRCAGRATCCCGGGGCCCRGGRGTTGCCCTCTCCCRRSTCTGGRGCRATAGGRRACCTTTCTGGC CCAGACAGEGTTAACAGTCCACATTCCAGAGCAGGGGAAAAAGGAGAGAGTCACAGACAAAAAGGGCCAGCTTCTAAC AACTTCACAGCTCTGGTAGGAGAGAGATAGATCACCCCCAACAATGGCCACAGCTGGTTTTGTCTGCCTCGAAGGAAACTGA GTGTGGGTGACAGAAGATGAAAA3GAGGCCAGGCAGAGATGGCCACAGATGGACGGGCACTTACAAGTGGAGGCAGGT3 ACAGOCTOCTTCCCAGTCTTCCTCCAGGGCTGGGGAGTCCTCCAAGCTTCTGTCTCAGTGCAGCTTCCGCCAGCCCCTCC 30 TECTTTTGCACCTCAGGTGTGAACCCTCCCTCCTCCTCCTCCTCCTGCGGCATGGCCCTCCTGCTACTGCAGGCTGAGCA AGGACCCAGAGCTGTTTGTGATACCATAAGAGGCTG3G3AGATGAT4TGGTAAGAGTGCTT3CTGTACAAGCAT3AAGAC ATGAGTTGGAATGCGGAAGGATGTGGAAAAATTATCTTTGTAACGTTGAGGGAAAGGGAGAGGTGGATTGTGG

AT GRIG SERVET URTOTOTOTOTORADARAT GRARATOT HT HT HT HT HAR SKA GROOT GROWAGE FRAGURA OT KLADDOT H GERGA DAS TRATARES DAS DATORESTEUS ES ATRABART DA DATORES TRATORES DAS TRATARES DA SER DATO DE SER DA SER DA S TTGCCTCTUUWWTGUTTTTGAAGABAARTTCAAGAGAGATCTCCTTGGTGATGAAATTGTAGGTGCTGAGAATUTGAAGA TBGGGT CRRTGGGATTCCTTCRRRGGGATGCTTCCCRGGGCTGGGTCATACTTCAATAGTRGGGAGGTGCTTGCAAGAAGAA GTGAGAGUUTAGGTTAGAGTOGDDAGAATOTGDDUUDAADDUUDQAAAAAGGDATDOTTUTGDDTCTGGGTGGGTGGGGT GAGCARRUR TOTTTAR CTARGACCATTAGCTGGCAGUUGTARCARATUR MUTTUGCTAGAGGARTTT MGT JARGTEGU T TODGO TO TOT THE WARREDUCK OTTSTOT DOTTT STOTARS CORRECTION OF STOTE TOTAL FRATISCUT CRESSION SOUR S GURGITARA NATTARRAGIURAGOT DATTITORITAT OT GRARA NURVAGADOTT GROOT GODDOTG (GRUSTANT) G GARAGOTAGOTATETOSOTECOTOROGRASASOSOSOSOSOSOSOROR ET COTORAGTOR COTAGERAGATECORAGORE ACCCCUST GREEK GREEK GREEK CATGES ACCASCCCT GOTO CASCCCCT COTTATTES CTUGGATGAGG CAGAGG GGGCTTTRARARGGCAROOGTPTCTRGGCTGGREENCTGGRGCCTGTPCTREUGRGTGCCCTCTTCCRUUTGGCRGCRTUC AGCCCT DACTA FOOCCGT GCCT CRTCT GCCTA CTTGT GCR CGCTGC TTT TTGT GCTGT GRAGGCCAGG GGTGGCAAR JC TTCA BRANTGATOC DA CAGAGAT DAT DOCAGOSTTT G BAGAGTACCUCBAGACT CUT DOT RABAA CHACKA BACKAT RAA GGGCAGGGACACATTTGCCTGGAAGAATACTAGCACAGCATTAGAACCTGGAGGGCAGCATTGGGGGGCTGGTAGAGAGC ACCCAAGSCAGGGTGGAGGCTGAGGTCAGCCSAAGCTGGCATTAACACGGGCATGGGCTGTATGATGGTGCAGAGAATC TCCTCCTAAGGATGAGACACAGGTCAGATCTAGCTGCTGACCAGTGGGGAAGTGATATGGTGAGGGATGCCAGATG 20 GGCTCCTGAGATABAGTCACCTGGGAGTABGAAGAGCTGAGACTGGAAGCTGGTTTGATCCAGATGCAAGGCAACCCTAG ATTGGGTTTGGGTGGGAACCTGAAGCCAGGAAGCCTTTAGTTCCCTTTAGTTGCCAGGGTCTSCTGAATGAGCCAGA GGGTTAGCATTALAXGAACAGGGTTTGTAGGTGGCATGTGACATGAGGGCAGCTGAGTGAATGTCGCCCTGTATGAGCA CAGGTGGCACCACTTGCCCTGAGCTTGCACCCTGACCCCAGCTTTGCCTCACTCCTGAGGACAGCACAGCACAGCACGACGACCA CAGCTGGASSGACACTCCAGAGARAATGACCTTGCTGGTCACCATTTSTSTGGGAGAGAGCTCATTTTCCAAGCTGGCCAC CGTTTTTGCAGAGGAAGTGCTTGACCTAAGGGCACTATTCTTGGAAAGCCCAAAACTAGTCCTTCCCTGGGCAAACAGG 30 TTATGTCATATTGATGCTGAGAGCATGGAACTTTTGGAGGTAGACAGGAGCCAGAGATGGATTAGTTAAAAGGCTGCGAT CCATCTAASCTCATGGTAGGAGATAGASCATGTCCAAGASAGGAGGGCAGGCATCAGACCTAGAAGATATGGCTGGGCAT ACAAGAGAGT OT GGTATACAGCAGGT GCTAAACAAATGCTTGTGGT AGCAAAAGCTATAGGTTTT GGGTCAGAACTCTGA

WO 00/32773

TIT BUBGGER STOUTCTOT OT HARMOT GER DET STORT REGES HRRTT ALKE DET WONDTTOGT HER TOTT HER FOUT ARTGRATTETTAT OU DIA UURO TIR OU CITTOTROCCO ROT CODENACARRANT ET CUTRATTIATIA LUTT CARTTARN OT COACTOCTTT OT COAT CTOCCT FRACACCCCCT TOCCAST FROM SAGOTARGRAD TOCACAGA SAGOTA SAGOA SAGOA SAGOA SAGOA SAGOA GT ST SGOTAGAG BOTAG 14 380A 3 B 3 OT 9G 9GATGA 3 PA SCTAAAC 7GGAA 3A 3T STTT 7 STTAGTA 3 2 TAGAAA 30 DTT SESTIGAGATUGCTASTAUCSSASAAGTSSAGATGGSOGCTGAGAAGTTGAAGAGCTAT (UATGCTTAACTAGAUAS), AGT TI SAGGUES TUTAS SUTACARARAN DA ANTONOSARAS SUTATUTATUT RATUUS SUDACIGARAN SUTACUCA SUNGA SUNGA SUNGA SUNGA SU ATAGTGGATGLAGTORTTGAATOOTGGAGGLAGUTATTTTAGAGATGTGAGAGALLAGGAAGTTTAAGTAGUUTGAGGC, G GATINA CARABARA STARGTGAKA SA SITTIDAGT STITIDAT DUOTGG STITIDAA SKACAGGRAGAGAGAAD JA SSKITIS S AT CTCRCTGCTCCCCGCTGCCTCCCTATARTCCATACAGATTCCGATAGCACGCCCGCAGGTTTGGAAAAAGAGATAACAGATAAA 10 GSTGSARAGAGAGACASTCTGSGCTAGGGTGCAGGCTCTCCCGAGACACCAGAATGTGTCCGAGAATGTGTCCAGAGACAGCT GOOGGERGOT SCHOT ACADOGGTT COTGERDEGERGOGGETGEOGGERGCOFFE SCOGGTGEGGERGTT GETGTGCTCC GROCHER STATES TO DE SECTION SECTION ACCIONACION CON CONTRACTOR SECTION SECTIO GTCTGGTGGCCTCFTGCAAGTGCAAGCGCCTCACCCCCTTCCACAACCAGTCGGAGCTCAAGACTTCGGGGAGACTC GOS DEGOCAÇARGOTOSCAR SOD ROGGOOGGCS DOGGGGAGAGO DAAGUAGGGGAGCTGGAGAAARGGCTA 15 GCCARACTTTCCAGAGCGTGTGGAGTTCCCAGCGCAGTAGAGACCGCAGGTGCTTCTGCCCGGTGGGGGGGAATGGGGAAG 3F3TGGGGTTC0CGC3GGCAGGAGAGGAAGCTTGAGTCDCAGACTCTGCCTAGCCCCGGGTGGGATGGGGATCTTTCTA CCCTCGCCGGACCTATACAGGACAAGGCAGTGTTTCCACCTTAAAGGGAAGGGAGTGTGGAACGAAAGACCTGGGACTGG TTATGGAGGTAGRGTAAGRTCTACTGCTTGGRGGGARATGTARAGGCTGGGTGGGTAGATAGGGTTTGTGAGGGTGRGG 20 TBBCCACTGAGTGTGATGTTGGGCTACGTGGTTCTCTTTTGGTACGGTCTTCTTTGTAAAATAGGGACCGGAACTCTGCT GREATTOCKAGGATTGGGGTACCCCCTGTAGACTGAGAGAGAGAGAGAACAGGGGAGGAGGGGTTAGGGGAGAGATTGTGG TORRATOTGCCTTCARATCCATATCTGGGATAGGGARAGGCGGGGTCCGAGAGATGGTGGARAGGGCCAGALATCACACTT CT3GCCCCCGGAAGAGCAGTGTCCCGGCCAACTGCCTT3TCATATTGTAAAGGGATTTTCTACACAACAGTTTAAAGG 25 GGGGGGGGGGGAGGGGAAGTGTTATACATATGCTGAGAAGGTGTCAGGGGGCACAGGACCACCACAATCTTTTTGT A-PATCATTTCCAGACACCTCTTACTTTCTGTGTAGATTTTAATTGTTAAARG33GAGGAGAGAGAGAGCGTTTGTAACAGAA GCACATGGAGGGGGGGGTAGGGGGGTTGGGGCTGAGTTTGGGGAAGTTTCCATGTGAGACTCATCCACAAAGACTGA AAGCCGCGTTTTTTTTTTAAGASTTCAGTGACATATTTATTTTCTCATTTAASTTATTTATGCCAACATTTTTTCTTS GGCATTGTTAATAAAGACAATGAATCTCGAGCAGGAGGSTGTGTGTTTTTTTTGTCAACCACACAATGTCTCGCCACT GTCATCTCACTCCCTTCCCTTGGTCACAAGACCCAARCCTTGACAACACCTCCGACTGCTCTGGTAGCCCTTGTGGCA ATACGTGTTTCCTTTGAAAAGTCACATTCATCCTTTCCTTTGCAAACCTGGCTCTCATTCCCCAGCTGGGTCATCGTCAT ACCOTCACCCCAGCCTOCCTTTAGCTGACCACTCTCCACCACTCTCTTCCAAAAGTGCACGTTTCACCGAGCCAGTTCCCT

GOT DAS AST AST DASS TOOCTOOT DATA AND AND ANGEST DI BUNK ASSA SKISTIGKT OF ANGEST CLAIF CASSA COU AST S S DUUT S CARCTAT C COTTOT CAST TABUT BAAT DTA STORUT SACA DUR CAT BAAT DOTT S COTTOT TA CACUMACTATTTGATTCCCARTTCTAGATCTTCCCTTGTTCATTCCTTGTGAGGATAGTCTCATCTCACCGAAGTCCT GOTT BATATTGGBATRARTGCRARGCBRGTACAATTGAGBADDRGTTBATCATTGGGCCRAGCTTTTT BRAAATGTGAL TETTACACUTATAGAASTGTARARSCCTTC:ARRGCASRGGCRATGCCTSSCTCTTCCTTCARCATGAGGGCTCCTGCTT TATGGGTUTGGTGGGTTAGTACATTCATAARTOCARCACTAGGGGTGTGAAASCAAGATGACTGGGGAGTTUGAGGCAAT ATGTGCACACTGGGGGTTGAACCTGGGCCTTTGTACCTGCGGGGGAAGCTGTCTACTGGTGTAAACCCAGCCGTCACTAC GREST COCTGTGGTTCCAGATTCCAGGAAGGACTTTTCAGGGAATCCAGGCATCCTGAAGAATGTCTTAGAGCAGGAGGA CAGGGTACTCAGGATTAAAAAGCTTCCCCCAAAACAATTUCAAGATCAGTTUCTGGTACTTGAGCGTGTTCAGCTATGCA 15 GAG DUCAGT GGGCATAGGTGAAGA DA DOGGTTGTA DTGTDAT GTACTAACT GTGCT TOAGAGGOUGUAGAGACAAATAAT GTTATRGTGRCCCCAGGGGACAGTGRTTCCAGAAGGRACACAGRAGAGAFTGCTGCTAGAGGCTGCCTGARGGAAAAGAG GTCCCAGA CTCTCTAAGCAAAGACTCCACTCACATAAAGACACAGGCTGA SCAGAGCTGGCCGTGGATGCAGGGAGGGGCCCA TOCACCATOCTTTAGCATGCCCTTGTATTCCCATCACATGCCAGGGGTGAGGGGCATCAGAGAGTCCAAGTGATGCCCAA ACCCARACACACCTAGGACTTGCTTTCTGGGACAGACAGATGCAGGAGAGACTAGGTTGGGCTGTGATCCCATTACCACA 20 RACARCAGGCTGATCTGGGAGGGGGTGGTRCTCTATGGCAGGGAGCACGTGTGCTTGGGGTACAGCCAGACACGGGGCTTG ATTCCTCCTCATARAGGAGACARAGTTGCAGARACCCARAGAGCCACAGGGTCCCCACTCTCTTGARATGACTTGGAC 25 TTGTTGCAGGGAAGACRGAGAGGGTCTGCAGAGGCTTCCTGGGTGACCCAGAGCACAGACACTGAAATCTGGTGCTGAGA COTGTATALACOCTOTTCCACAGGTTCCCTGAAAGGAGCCCACATTCCCCAAACCCTGTCTCCTGACCACTGAGGATGAGA GCACTTGGGGCTTCCCCATTCTTGGAGTGCACCTGGTTTCCCCCATCTGAGGGCACATGAGGTCTCAGGTCTTGGGAAAA GTGTAGCRTTTACAAGCCTGGTGCCTGAGGRGATCAGARGRTGGCRTCAGRTACCCTGGRACTGGRCTTGCAGACAGTTA 30 CGTAACGTGAGACTAGGGCAGGGTGATCCCCCCAGGGCAGGCCGATGGCCCTGTGTAGTTATTAGCAGCTGTAGTCTTATTC CTTARTAASTCCCAGTTTGGGGCAGGAGATATGTATTCCCTGCTTTGARGTGGCGGGCCAGTTATCTACTTCCAAGT TTTCCCTGAGCAGTCAGGCCAGTCCAAA3CCCTTCAATTTAGCTTTCATAAGGAACACCCCTTTTSTTGGGTGGAGGTAG

CACTITO UCITORATICUCAR CATORA RAR RECIAMAMA CRIST URBANTOT UTURO AR RITO DE 1920 DE 2001T E TOUTA URBARAT SARTTODAAGAGAGOUAGGOTADAGAGAAAAGGGTSTGTOWAAAAAAAAGAGGAAGAAGAAGAAGAATAAA RAAAAAGAAGAGAGAGAGAGAAGAAGAAGAAGAAGAA CRREAR OVER CREARCH SERRER CRAGG CROAGESTITT STUDUUSTRITTTATTRATCRITETTTT STUDOTTT SUCRITT TA BACTANAAGACT OBGWAAAG GAGGTCTCTCTGTGTTTCTCATCCGGGACACACCCGAGAAGATGTAT BBAAGATGT THAT FTOCTO CASTTGCACATOT SEGGOTGG STOGATTEGTTAGATS SCATOSUCTGG ST STGGTTACSAT SACTOGCS AMBAR BUR STAT FTØGT SCATA FORAR DØRÆGGRASTITG DRICAGRASTAR DA DT STØT STATT SATGT SDA SUTAT NGONS CAT BOARGCRARAGCCRARGGRACAGOCTTRAGATRATGTTTCCRCRARACCCCTCCCCCCTTTTRACATGGGCCTTTTTCA TTBYUUTGGAGOTTGOCARCTGGGCTGGGCTGGCTABUTTGTAGGTOULAGGATOTGGATATGTGTGCCTGCCTAATGT GRORAT ROOT GARTGACTROTTCATCTOCCARROCCTBGGRTTCTACTTTCTACTARRYTATTCTATTRARTCARTG 10 ASSOCIATISTOSSTAGRACASTTUTTAGGOCTOCTGAGAGTCARSTISSGAGTGAGAGCAAGCCTGGAGROCCATC RECERRECENTEGRACIALGRART CLARACTIGGGRIT DURGGCICGGGRITATGGRIRTACAGRARGGGTCASGGRAGGAL AT BRACCAGATGRATAGAGGCAGGRAGGTAGGGCUCTGCATACATGGRACCTGGTGTACATGTTATCTGCATGGGGGTTT GCATTGCAAT BUGTOTTCAGCAGGTTCACCACACTGGGAAACAGAAAACCARAAA RAAGAGTAGGTGTTGGAGTCAGA TACTGTCAGTUATGCCTGAAGAAATGGAAGCAATTAACGATGCGCCGCAATTAGGATATTAGCTCCCTGAAGAAAAAGCAA GRAGCTGGGCTGTGGGCACTGRAGGGAGCTTTGRATGRTGTCACATTCTCTGTATGCCTACGAGGGCAGTATTGGAGACT GAGACTTGACTTGTGTGCCATATGATTGCTCCTTTTCCTACAGTCATCTGGGGCTCCTGAGCTTCGTCCTTJTCCAAGA ACCTUGAGCTGGCAGTGGGCAGCTGATAGATGTCTGCAAGAATATCTGAAAAGAGGGGGAAGATGAAGGAGGACCC AGAGRACUACURACOTROCTGACARAGOTGCAGGACCAGTCTCTCCTACAGATGGGAGACAGAGGCGAGAGATGA 20 ATGGTCRGGGGAGAGTCAGAGARAGGAGAGAGGGTGAGGCAGAGAGAGCCRAAGGAGGARACACTTGTGGTGTACAGCTACTACT ACTGASTACCAGCTGCGTGGCAGACAGCCAATGCCAAGGCTCGGCTGATCATGGCACCTCGTGGGAACTCCTAGCCAGTG CTCCTAAGTGCTGGAATTAARGGCCTGCGGCCACCACTGCCGGCCTAARGCTACTTTAAGAGAGAGAGAGAATGTATAAG 25 TATTATAATTOORGGTTATAGTTORTTGCTSTAGARTTGGAGTOTTORTATTOORGGTAATCTOOCAGAGAGACATGOCAGA AAACAR COTGTTCTACGAAATCTCTCATGGACTCCCTTCCCCAGTARTTCTARACTGTGTCAAATCTACARGAAATAGTG ACAGTCACAGTCTCTAACGTTTTGGGCATGACTCTGARGTCTCATTGCTAAGTACTGGGAAGATGAAAACTTTACCTAGT GTCAGCATTTGGAGCAGAGCCTTTGGGATTTGAGATGGTCTTTTGCAGAGCCTACTAGTACATGGAGAGAGGGGGCC 30 GGATCACCCACCTTGCACCTCCAGAACTCAGAGCCAARTTAAACTTTCTTGTTACTGTCGTCAAAGCACAGTCGGTC TOOTHTAGAGAAAAGAAAATTAGOTGGCACACAGATAGAGGCCCTGGAGGAGGCTGGCTTGTCCTTGTCCCCGAGGAGGTG GCGAGTAAGGTGTAAATGTTCATGGATGTAAATGGGCCCATATATGAGGGTCTGGGGTAACAAGAAGGCCTGTGAATATA

WO 00/32773

ACTION OF APTICATION OF ANTICACED ANTICACED ANTICATION OF THE RETURNARIAN CARACTERS AND CARACTERS AN CATALORA (CA JACE STOWS TO TO TO TO TO TO TO TO TALABOUT OF TO SETTE SOTA STANDARD AS SOLD SET TRUARGE RESTABLATATION DARGOOT RAARTOONT STARRING GOATRY ON ON SARARTITUR RAARST SATUTRY RE TOCAGGACIS INTERACTOR CONTINUO CON CONCERCA SACTORA SETURBA SETURBA SETURBA SETURBA CONTINUO TOCTOCOUTY OF THE PROPERTY OF THE PROPERTY OF A CONTRACT AND A CONTRACT SOUTH OF THE PROPERTY SOUTH OF THE PROPERTY OF THE PRO AUTOSCONA LE PUT ETT ACTICITERACTETTICARACHATI UT STOTOTOCAMAT PETTAMITA TUT PUT NI UN TONTO NAC COTOGET INSUTTOTTOCTTOGRASTOSCOCT CASTSTOTCTARGET SATIOTTOTARGATATTOTTTITARRAS SOT JABJA (TO STOODAUT UTGOSAGTTURRRGOORGOOTGATUTACRORGORGORGOTOORGGATRTUCAGGOORGTOTTUGGARARAAGGT TT CT CRAR CRARRAGRAGT CRETT ST CAGGAGGAGGAGGAGGAGT PAGRETCTAGA (GRÖCCAT GETGAT GCATA COTTTOATO JARGOROTTAGGRASCARAGARAGGTGARACTOTTTGACTTTGAGGCAGGTAGGTTS CATAGTGATACCC TECTTA STOT STOTETST STOTETSTOTETSTOTETSTOTETSTOTETALLTTTLALABTOTELLALTSCATTOTTLALLA ACTICUSTAGAS TRAGACTICATAGA CAGTTOTGA CACTICO COAR DOCCUMACO ATIGTO GOTGUTTGARGOTARACTICOT ST COTTTGTARASCAGCAGGTGTCTATGRACCCTGARCCATCTCTCCAGTCTCCAGATGTGCATTCTCRAAGAGGAGTCCTT CATATTT DOUTARACTGARCATCCTTATCAGTGAGCATCCTCGAGTGACCARAGCTACTGCARACCCTCTTAGGCARACRT CARAAGCATGCATGTACACCATTCTTATTAGACTATGCTTTGCTARAAGACTTTGCTAGATACTTTAAAACATCACTTGT GCCTTTTGGTPGGGAGGTTCCAAGATTGGTACTGGCGTACTGGAAACTGAAAGGTAGAGATCTAGAAATCACAGGCAG TORGRAGGSCORGOCTGTACAAGAGAGAGAGTTOCACACOTTCCAGGAACACTGAGCAGGGGGGGGCTGGGACCTTGCCTCAG 20 CODAAGAAR DTAGTGCGTTTCCTGTRTGCRTGCCTCTCAGAGATTCCRTARGATCTGCCTTCT30CATAAGATCTCCT3C ATCCAGACAAGCCTAGGGGAAGTTGAGAGGCTGCCTGAGTCTCTCCCACAGGCCCCTTCTTGCCTGGCAGTATTTTTTTA ARCTGATCTAGGGAGCTGGGTCAGCAGTTAAGAGTTCTGGGTGGCCTTGCTTCAGATCTTGGTTTGATTCGCAGCACCCA CATGATGGCTTTCAACTGTATCTCTGCTTCCAGGGGATCCAACAGCCTCTTCTGACCTCCATAGACAAGACCTAGTCCTC 25 TGCRAGAGCACCARATGCTCTTATCTGTTGATCCATCTCTCTAGCCTCATGCCAGATCATTTAARACTACTGGACACTGT TTTATAAGA-AGATATCTGCATTTGTCTCCTGAGAGAACAAAGGGTGGAGGGCTACTGAGATGGCTCTAGGGGTAAAGGT CCTCAPACTTCCCACACATGTGCTGTGGCTTATGTGTARCCCCAATARGTAPAGATAGTTTTAPACACTACATAAGGTAG 30 GGTTTCTTCATGACCCCAAGGAATGATGCCCCTGATAGAGCTTATGCTGAAACCCCATCTCCATTGTGCCATCTGGAAAG AGRICARTT SCAT COORGARA CAGART STT CAT SART GGAT TAAT GAGCT AT TAAGARAGT G SCT TGGT TAT TGCACAT GC TGGCGGCGTAATGACCTCCACCATGATGTTATCCAGCATGAAGGTCCTCACCAGAAGTCATACAAATCTTCTTAGGCTTC CAGAGTOGTGAGCABARRAGGAGAGTOTTARATARATTARGTAGGGTUAGGTAGTTARGGAGGGARRATGARGGARAGG 35 AGTTCTARTACRARACCROTTCCCTTCCCTGTTCARACCRCAGTGCCCTRTTATCTARACARRACTTCARGCCRAGCT TTTAGGTTGCCAGTATTTATGTAACAACAAGGCCGGTTGACACACATCTGTAACTCGTAGTACTGGGGCTCAGGGGCAGA

GAIGN BOT GRAG CONTROLACTITY RARTT CONGRETT OT STIBNORARD TOT STICHARA BRONATATO STIGNOT BAICCOURS ABBRITATUT MATRITURAUTT UTGEODRAGAGAGAGAGETOTOTOGORGAT IT MTASTTBURAG COTTTTEGAGTAR MTTT A SCCARACTCASACTTTGCAAGTCTTTGTGGACTGAACTGCACGTGTT3CTCSTGATCTACAAAGTCACCCTCCTTCTCAAG CTAGCAGCACTGGGTTAGGGCCAGCTGCTCATTGAAGGCCTCTTTGCAGAGTATCACGGGGGGAGCAGGAGGAGCAGCAGCTC COTAGAACACCAGOCTGTUGTUGTUTATTGAGGACATTATTGAGGGCUAAGATGAGAGATAACTCTGTCACCTGTCACCAGAACAA CAGTOWGGTSTTGCGGTGTTAGGTTATTTCTGTGTCTCTGCAGAARAGAYTG/AAGCTGWAGAAAAGARATAAATGATATC TTTTTCATTCRGCARCTAGATTCCFTGGTACARLAGGTCCCTGGGGARAGGCGGGGGGAGACGCGGGGGCTCCTGARCTC STOTGTGTACTCRCRGGGAGGAGGTTGGCARAGCCCTGGTCCTCTACMGGTGGAGAAGGGGGAAGCTGTCGGTCGACCTGT TWACTTITTTWCCTTTCTCTTTTCTTAGAAACCASTCTCAATTTAAGATAATGAGTCTCCCCATTCACGTGTSCTCACT ARRATGTGGCTGGACCGTGTGCGGCACGARACCAGAGATTGGCGGTCTARGTTACATGCTCTCTGCCAGCCCGGGTGCCT TTTCTTTTCGGAAAAGGAGACCCGGGAGGTAAAAGGAAGTTTGCCAACTTTTGATGATGTGTGCGCGGGGTGACTCTTTAAA ATGTICAT CONTACCTGGGATAGGGAAGGCTITTICAGGGAAGTCATCTAGCCTICCCTTCAGGAAAAAGATTICCCGTT TTAUTTAGOTTCCACCTGGTDCCTTATCCGCTGTCTCTGUCCACTAGTUCTCATCCATCUGGTTTCCGCCUTCATCCACC 15 TTGCCCTTTTAGTTCCTAGARAGCR3CACCGTAGTCTTGGCAGGTGGGCGATT3GTCACTGCGCTACCACTGTTACCATG TGAGAACTGGAGTTCAATTCCCAGCACCATGGATGTATTTCCAGCACCTGGAAGGCAGGAGGAGGAGATCTTAAAGCTCCT GGCCAGACAS DOCAGCCTARTTRGTARTCAGTGAGAGACCCCTGTCTCAAGAAAAAACATGGAACATCAAAGSTCAACCTS 20 GOTOTTSTOACCCCCACTAAGGCTTCAACTTCTTCTRTTTCTTCATCTTGACTCCTGTACTTTGCATGCCTTTTCCAG CARAGGETTTTOTTTAAATOTEGGTCATTGATAAROTECSTOTARATTTOTTCCSCTGGCCTTTTCTTTCTGTGTAGGGA GRTRARGRICACROTACARAGTORODGTGGGRCCRGTTTATTCRCCCRDCCRCCCCTGCTTCTGTTCATDCGGCCRGCT ARGTAGTCCARCCTCTCTGGTGCTGTACCCTGGACCCTGGCTTCACCACAGCTCCTCCATGCTACCCAGCCCTGCAAAACC TTCAGCCTAGCCTCTGGTTCTCCAACCAGCACAGGCCCAGTCTGGCTTCTATGTCCTAGAAATCTCCTTCATTCTCTCCA TTTCCCTCCTGAATCTACCACCTTCTTTCTCCCTTCTCCTGACCTCTAATGTCTTGGTCAAACGATTACAAGGAAAGCAA CCGGATTCTGACAGCAGTTCCGAAGCTGAGTCCAGGAAGCTGAATTTAAAATCACACTCCAGCTGGGTTCTGAGGCAGC 30 GTGGTGGTGGTGGTGGTGGTGGTGTGTGTGTGTTTTTCTGCTTTTACAAAACTTTTCTAATTCTTATACAAA GACARATOTGCCTCATATAGGCAGRAAGATGACTTATGCCTATATAAGATATARAGATGACTTTATGCCACTTATTAGCA ATAGTTACTGTCARRAGTARTTCTATTTATACACCCTTATACATGGTATTGCTTTTGTTGGAGACTCTARRATCCAGRTT ATGTATTTALLLLLLATTCCCCASTCCTTALLAGGTSAGARCTGGAGATAGARGTCAGGGCACLAGTATGGAGT CGGAGTGTGGAGTCCTGCCAATGGTCTGGACAGAAGCATCCAGAGAGGGTCCAAAAGAAATGCCTCGCCTCCTAAGGAAC 35 ACTGGCAGCCCTGATGAGGTACCAGAGATTGCTAAGTGGAGGAATACAGGATCAGACCCATGGAGGGGCTTAAAGCGTGA

OT STAG JA S DOUT TO SOT GAGGGSOT OCARST SOCIOCOLARSOTS CTG CART SISASONA TATGA SAGGT SATISTICT S RARECTITICS TABBARCTICCERS DATE SET TOTS OT BITTOS SEGENTE ADUBATITO TOTAL DATE DE UTOTA DARGE PER CARDA TOTAL DA CA ATS STTRATSASTTSGRACTARARCAGGGGCCATCACACTGSCTCCCATAGCTCTGGCCTTGCCACCTTCCACATCTGC TODDACODD TTST TTGGCACCACCTCAAGCT DTGTGACTCTACACAT DCAAAAGAGAAGAGAAGAGTACCCTACTGGGCATGCC ACCTOTTOTTOAGCATCAGGTGAGAGTGTGGCAAGCCTAGGCTCCTCTCGAGGTGCAGGGTGCCAGAATAGGATAGGATAGGATGCTC ARCTATOTOCTGAGCTGGAACTATTTTAGGAATAAGBATTATBCCCGTCGGGGGTTGGCCAGCACCCCAGCAGCAGCTGTGC TT KOSTAAAAVCAASTSCTGTTSATTTAT OTAAAAACASAGUDGTGGACCDACCCACAGGA JAAGTAT KTATGCAT OT ST TTBAGAGAGGGTTTCTCTGTGTAGTCCTGGGTGTCTTGGAACTCAGTTGTAGAGGAGGGTGGGCTCGAAACTCAGAAATC CTGGGATTARAGTGTGTGCACCACGCCCGGCCCTARCCCCCRTTCTTARTGGTGATCCRGTGGAARTTTCGGGGCC 10 ACACACATGTCCATTAGGGATTAGCTGCTGTCTTCTGAGCTACCTGGTACAATCTTTATCCCCTGGGGCCTGGGCTCCTG ASCTOGATTASCTCATCCTGGCTGGCCTGGCCTGTTCTTACTTACACTCTTCCCCTTGCTCTGGACTTGTTTCTTTA OT DRAGTOST OT GCCACAGT CCCTAAGCCACCT OT GTAAGACAACTAAGAT ACTTCCCT CAAGCACGAAAGCACCTCC AGTCACCACACCCTCTGGAGGTGTGTGGACACATGTTCATGCGTUTGGTTGCGCTTACGTACGTGCGC 15

Sequence ID No. 18: Human Beer Genomic Sequence (This gene has two exons, at positions 161-427 abd 3186-5219).

20

25

30

aacttotott tgggaggott ggaagactgg ggtagaccca gtgaagattg ctggcototg 540 ccagcactgg tcgaggaaca gtcttgcctg gaggtggggg aagaatggct cgctggtgca 600 gccttcaaat tcaggtgcag aggcatgagg caacagacgc tggtgagagc ccagggcagg 660 5 gaggacgctg gggtggtgag ggtatggcat cagggcatca gaacaggctc aggggctcag 720 aaaagaaaag gtttcaaaga atctcctcct gggaatatag gagccacgtc cagctgctgg 780 10 taccactggg aagggaacaa ggtaagggag ceteccatee acagaacage acetgtgggg 840 caccggacac totatgotgg tggtggctgt coccaccaca cagacccaca toatggaato 900 15 cccaggaggt gaacccccag ctcgaagggg aagaaacagg ttccaggcac tcagtaactt 960 ggtagtgaga agagctgagg tgtgaacctg gtttgatcca actgcaagat agccctggtg 1020 tgtgggggg tgtgggggac agatetecae aaageagtgg ggaggaagge cagagaggea 1080 20 cccctgcagt gtgcattgcc catggcctgc ccagggagct ggcacttgaa ggaatgggag 1140 ttttcggcac agttttagcc cctgacatgg gtgcagctga gtccaggccc tggaggggag 1200 agcagcatcc tctgtgcagg agtagggaca tctgtcctca gcagccaccc cagtcccaac 1260 25 cttgcctcat tccaggggag ggagaaggaa gaggaaccct gggttcctgg tcaggcctgc 1320 acagagaagc ccaggtgaca gtgtgcatct ggctctataa ttggcaggaa tcctgaggcc 1380 30 atggggggt ctgaaatgac acttcagact aagagcttcc ctgtcctctg gccattatcc 1440 aggtggcaga gaagtccact gcccaggctc ctggacccca gccctccccg cctcacaacc 1500 35 tgttgggact atggggtgct aaaaagggca actgcatggg aggccagcca ggaccctccg 1560

tetteaaaat ggaggacaag ggegeeteee eesacagete eesttetagg caaggteage 1620 tgggctccag cgactgcctg aagggctgta aggaacccaa acacaaaatg tccaccttgc 1680 tggactecca egagaggeca cageecetga ggaagecaca tgeteaaaac aaagteatga 1740 tetgeagagg aagtgeetgg cetaggggeg etattetega aaageegeaa aatgeeeet 1800 tecetgggea aatgeeece tgaceacaca cacattecag eeetgeagag gtgaggatge 1860 10 aaaccagece acagaccaga aagcagecee agacgatgge agtggecaca teteceetge 1920 tgtgcttgct cttcagagtg ggggtggggg gtggccttct ctgtcccctc tctggtttgg 1980 15 tottaagact atttttcatt otttottgto acattggaac tatooccatg aaacotttgg 2040 gggtggactg gtactcacac gacgaccage tatttaaaaa geteecacee atetaagtee 2100 accataggag acatggtcaa ggtgtgtgca ggggatcagg ccaggcctcg gagcccaatc 2160 20 tetgeetgee cagggagtat caccatgagg egeceattea gataacacag aacaagaaat 2220 gtgcccagca gagagccagg tcaatgtttg tggcagctga acctgtaggt tttgggtcag 2280 agctcagggc ccctatggta ggaaagtaac gacagtaaaa agcagccctc agctccatcc 2340 25 cccagcccag cctcccatgg atgctcgaac gcagagcctc cactcttgcc ggagccaaaa 2400 ggtgctggga ccccagggaa gtggagtccg gagatgcagc ccagcctttt gggcaagttc 2460 30 ttttctctgg ctgggcctca gtattctcat tgataatgag ggggttggac acactgcctt 2520 tgattccttt caagtctaat gaattcctgt cctgatcacc tccccttcag tccctcgcct 2580 ccacagcage tgccctgatt tattacctte aattaaccte tactcettte tecatecect 2640 35

gtccacccct cccaagtggc tggaaaagga atttgggaga agccagagcc aggcagaagg 2700 tgtgctgagt acttaccctg cccaggccag ggaccctgcg gcacaagtgt ggcttaaatc 2760 ataagaagac cccagaagag aaatgataat aataatacat aacagccgac gctttcagct 2820 5 atatgtgcca aatggtattt tetgeattge gtgtgtaatg gattaaeteg caatgettgg 2880 ggcggcccat tttgcagaca ggaagaagag agaggttaag gaacttgccc aagatgacac 2940 10 ctgcagtgag cgatggagcc ctggtgtttg aaccccagca gtcatttggc tccgagggga 3000 cagggtgcgc aggagagctt tccaccagct ctagagcatc tgggaccttc ctgcaataga 3060 tgttcagggg caaaagcctc tggagacagg cttggcaaaa gcagggctgg ggtggagaga 3120 15 gacgggccgg tccagggcag gggtggccag gcgggcggcc accctcacgc gcgcctctct 3180 ccacagacgt grccgagtac agcrgccgcg agcrgcactt cacccgcrac grgaccgarg 3240 20 ggccgtgccg cagcgccaag ccggtcaccg agctggtgtg ctccggccag tgcggcccgg 3300 cgcgcctgct gcccaacgcc atcggccgcg gcaagtggtg gcgacctagt gggcccgact 3360 teegetgeat eccegacege tacegegege agegegtgea getgetgtgt eccggtggtg 3420 25 aggegeegeg egegegeaag gtgegeetgg tggeetegtg caagtgeaag egeeteacee 3480 gcttccacaa ccagtcggag ctcaaggact tcgggaccga ggccgctcgg ccgcagaagg 3540 30 gccggaagcc gcggccccgc gcccggagcg ccaaagccaa ccaggccgag ctggagaacg 3600 cctactagag cccgcccgcg cccctcccca ccggcgggcg ccccggccct gaacccgcgc 3660 cccacatttc tgtcctctgc gcgtggtttg attgtttata tttcattgta aatgcctgca 3720 35

acccagggca gggggctgag accttccagg ccctgaggaa tcccgggcgc cggcaaggcc 3780 cccctcagec cgccagetga ggggtcccae ggggcagggg agggaattga gagtcacaga 3840 cactgageca egeageceeg cetetgggge egectacett tgetggteee actteagagg 3900 aggcagaaat ggaagcattt tcaccgccct ggggttttaa gggagcggtg tgggagtggg 3960 aaagtccagg gactggttaa gaaagttgga taagattccc ccttgcacct cgctgcccat 4020 10 cagaaageet gaggegtgee cagageacaa gaetggggge aactgtagat gtggttteta 4080 gtcctggctc tgccactaac ttgctgtgta accttgaact acacaattct ccttcgggac 4140 ctcaatttcc actttgtaaa atgagggtgg aggtgggaat aggatctcga ggagactatt 4200 15 ggcatatgat tecaaggaet ecagtgeett ttgaatggge agaggtgaga gagagagaga 4260 gaaagagaga gaatgaatge agttgcattg attcagtgce aaggtcactt ccagaattca 4320 20 gagttgtgat gctctcttct gacagccaaa gatgaaaaac aaacagaaaa aaaaaagtaa 4380 agagtctatt tatggctgac atatttacgg ctgacaaact cctggaagaa gctatgctgc 4440 ttcccagcct ggcttccccg gatgtttggc tacctccacc cctccatctc aaagaaataa 4500 25 catcatccat tggggtagaa aaggagaggg tccgagggtg gtgggaggga tagaaatcac 4560 atcogococa acttoccaaa gagoagoato octococoga cocatagoca tgttttaaag 4620 30 tcaccttccg aagagaagtg aaaggttcaa ggacactggc cttgcaggcc cgagggagca 4680 gccatcacaa actcacagac cagcacatcc cttttgagac accgccttct gcccaccact 4740 35 cacggacaca tttctgccta gaaaacagct tcttactgct cttacatgtg atggcatatc 4800

rtacactaaa agaatattat tgggggaaaa actacaagtg ctgtacatat gctgagaaac 4860 tgcagagcat aatagctgcc acccaaaaat ctttttgaaa atcatttcca gacaacctct 4920 tactttctgt gtagttttta attgttaaaa aaaaaaagtt ttaaacagaa gcacatgaca 4980 5 tatgaaagcc tgcaggactg gtcgttttt tggcaattet tecaegtggg acttgtecae 5040 aagaatgaaa gtagtggttt ttaaagagtt aagttacata tttattttct cacttaagtt 5100 10 attratgcaa aagtttttct tgtagagaat gacaatgtta atattgcttt atgaattaac 5160 agtotgttot tocagagtoc agagacattg ttaataaaga caatgaatca tgaccgaaag 5220 gatgtggtct cattitgtca accacacatg acgicattic tgtcaaagtt gacaccctic 5280 15 tettggteae tagageteea acettggaea cacetttgae tgetetetgg tggeeettgt 5340 ggcaattatg tottootttg aaaagtcatg tttatccctt cotttccaaa cocagaccgc 5400 20 atttcttcac ccagggcatg gtaataacct cagccttgta tccttttagc agcctcccct 5460 ccatgctggc ttccaaaatg ctgttctcat tgtatcactc ccctgctcaa aagccttcca 5520 tagctccccc ttgcccagga tcaagtgcag tttccctatc tgacatggga ggccttctct 5580 gettgaetee caceteecae tecaceaage tteetaetga etceaaatgg teatgeagat 5640 ccctgcttcc ttagtttgcc atccacactt agcaccccca ataactaatc ctctttcttt 5700 30 aggattcaca ttacttgtca tctcttcccc taaccttcca gagatgttcc aatctcccat 5760 gatccctctc tcctctgagg ttccagcccc ttttgtctac accactactt tggttcctaa 5820 ttctgttttc catttgacag tcattcatgg aggaccagcc tggccaagtc ctgcttagta 5880 35

otggoataga caacacaaag coaagtacaa ttoaggacoa gotoacagga aacttoatot 5940 tettegaagt gtggatttga tgeeteetgg gtagaaatgt aggatettea aaagtgggee 6000 5 agestectigs actististica aagteteges tesseaaggt gistiaatag igsiggaigs 6060 tagctgagtt agcatettea gatgaagagt aaccetaaag ttactettea gtigecetaa 6120 ggtgggatgg tcaactggaa agctttaaat taagtccagc ctaccttggg ggaacccacc 6180 10 cccacaaaga aagctgaggt ccctcctgat gacttgtcag tttaactacc aataacccac 6240 ttgaattaat catcatcatc aagtetttga taggtgtgag tgggtatcag tggccggtcc 6300 15 cttcctgggg ctccagccc cgaggaggcc tcagtgagcc cctgcagaaa atccatgcat 6360 catgagtgtc tcagggccca gaatatgaga gcaggtagga aacagagaca tcttccatcc 6420 ctgagaggca gtgcggtcca gtgggtgggg acacgggctc tgggtcaggt ttgtgttgtt 6480 20 tgtttgtttg ttttgagaca gagtctcgct ctattgccca ggctggagtg cagtgtcaca 6540 atctcggctt actgcaactt ctgccttccc ggattcaagt gattctcctg cctcagcctc 6600 cagagraget gggattaeag grgcgrgeea ceaegeergg etaattring tarringat 6660 agagacgggg tttcaccatg ttggccaggc tagtctcgaa ctcttgacct caagtgatct 6720 geotgeeteg geoteceaaa gtgetgggat tacaggegtg agecaceaca cecageecca 6780 30 ggttggtgtt tgaatctgag gagactgaag caccaagggg ttaaatgttt tgcccacagc 6840 catacttggg ctcagttcct tgccctaccc ctcacttgag ctgcttagaa cctggtgggc 6900 acatgggcaa taaccaggtc acactgtttt gtaccaagtg ttatgggaat ccaagatagg 6960 35

agtaatttgc totgtggagg ggatgaggga tagtggttag ggaaagcttc acaaagtggg 7020 tgttgcttag agattttcca ggtggagaag ggggcttcta ggcagaaggc atagcccaag 7080 caaagactgc aagtgcatgg ctgctcatgg gtagaagaga atccaccatt cctcaacatg 7140 taccgagtee tigecatgig caaggeaaca igggggtace aggaatteea ageaatgiee 7200 aaacctaggg tetgetttet gggacetgaa gatacaggat ggateageee aggetgcaat 7260 10 cccattacca cgagggggaa aaaaacctga aggctaaatt gtaggtcggg ttagaggtta 7320 tttatggaaa gttatattot acctacatgg ggtctataag cctggcgcca atcagaaaag 7380 gaacaaacaa cagacctagc tgggaggggc agcattttgt tgtagggggc ggggcacatg 7440 15 ttctgggggt acagccagac tcagggcttg tattaatagt ctgagagtaa gacagacaga 7500 gggatagaag gaaataggte eetttetete tetetete tetetete aetetete 7560 20 teteteacae acaeacaea acaeacaea aegetetgta ggggtetaet tatgeteeaa 7620 gtacaaatca ggccacattt acacaaggag gtaaaggaaa agaacgttgg aggagccaca 7680 ggaccccaaa attccctgtt ttccttgaat caggcaggac ttacgcagct gggagggtgg 7740 25 agageetgea gaageeacet gegagtaage caagtteaga gteacagaea eeaaaagetg 7800 gtgccatgtc ccacaccege ccacetecca ectgeteett gacacageee tgtgeteeae 7860 30 aacceggete ceagateatt gattataget etggggeetg cacegteett eetgecacat 7920 ccccacccca ttcttggaac ctgccctctg tcttctccct tgtccaaggg caggcaaggg 7980 ctcagctatt gggcagcttt gaccaacagc tgaggctcct tttgtggctg gagatgcagg 8040 35

aggcagggga atattectet tagtcaatge gaccatgtge etggtttgee cagggtggte 8100 togettacae eegeaggeea agegtaatta ttaacagete ceaettetae tetaaaaaat 3160 5 gacccaatit gggcagtaaa tiataiggig cocatgotai taagagcigc aactigcigg \$220 gegtggtgge teacacetgt aateeeagta etttgggaeg teaaggeggg tggateacet 8280 gaggicacga gitagagaci ggcciggcca gcaiggcaaa accccaicii tactaaaaai 8340 10 acaaaaatta gcaaggcatg gtggcatgca cctgtaatc: caggtactcg ggaggctgag 8400 acaggagaat ggcttgaacc caggaggcag aggttgcagt gagccaagat tgtgccactg 8460 ccctccagcc ctggcaacag agcaagactt catctcaaaa gaaaaaggat actgtcaatc 8520 15 actgcaggaa gaacccaggt aatgaatgag gagaagagag gggctgagtc accatagtgg 8580 cagcaccgac teetgeagga aaggegagae aetgggteat gggtaetgaa gggtgeeetg 8640 20 aatgacgttc tgctttagag accgaacctg agccctgaaa gtgcatgcct gttcatgggt 8700 gagagactaa atteateatt eettggeagg taetgaatee tttettaegg etgeeeteea 8760 atgeccaatt teestacaat tgtetggggt geetaagett etgeccaeca agagggeeag 8820 agctggcagc gagcagctgc aggtaggaga gataggtacc cataagggag gtgggaaaga 8880 gagatggaag gagagggtg cagagcacac acctcccctg cctgacaact tcctgagggc 8940 30 tggtcatgcc agcagattta aggcggaggc aggggagatg gggcgggaga ggaagtgaaa 9000 aaggagaggg tggggatgga gaggaagaga gggtgatcat tcattcattc cattgctact 9060 gactggatgc cagctgtgag ccaggcacca ccctagctct gggcatgtgg ttgtaatctt 9120 35

ggagcctcat ggagctcaca gggagtgctg gcaaggagat ggataatgga cggataacaa 9180
ataaacattt agtacaatgt ccgggaatgg aaagttctcg aaagaaaaat aaagctggtg 9240
agcatataga cagcctgaa ggcggccagg ccaggcattt ctgaggaggt ggcatttgag 9300
c

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

CLAIMS

We claim

- 1. An isolated nucleic acid molecule selected from the group consisting of:
- (a) an isolated nucleic acid molecule comprising sequence ID Nos., 1, 5, 9, 11, 13, or, 15, or complementary sequence thereof;
- (b) an isolated nucleic acid molecule that specifically hybridizes to the nucleic acid molecule of (a) under conditions of high stringency; and
- (c) an isolated nucleic acid that encodes a TGF-beta binding-protein according to (a) or (b).
 - 2. The isolated nucleic acid molecule according to claim 1 wherein said nucleic acid molecule encodes a protein comprising the protein of Sequence ID NO. 2.
- 3. The isolated nucleic acid molecule according to claim 1 wherein said nucleic acid molecule encodes a protein comprising the protein of Sequence ID NO. 6.
 - 4. The isolated nucleic acid molecule according to claim 1 wherein said nucleic acid molecule encodes a protein comprising the protein of Sequence ID NO. 10.
- 5. The isolated nucleic acid molecule according to claim 1 wherein said nucleic acid molecule encodes a protein comprising the protein of Sequence ID NO. 12.
- 6. The isolated nucleic acid molecule according to claim 1 wherein said nucleic acid molecule encodes a protein comprising the protein of Sequence ID NO. 14.
 - 7. The isolated nucleic acid molecule according to claim 1 wherein said nucleic acid molecule encodes a protein comprising the protein of Sequence ID NO. 16.

- 8. An expression vector, comprising a promoter operably linked to a nucleic acid molecule according to any one of claims 1 to 7.
- 9. The expression vector according to claim 8 wherein said promoter is selected from the group consisting of CMV I-E promoter, SV40 early promoter and MuLV LTR.
- 10. The expression vector according to claim 8 wherein said promoter is a tissue-specific promoter.
- 11. A method of producing a TGF-beta binding protein, comprising, culturing a cell which contains a vector according to claim 8 under conditions and for a time sufficient to produce said protein.
 - 12. The method according to claim 11, further comprising the step of purifying said protein.
 - 13. A viral vector capable of directing the expression of a nucleic acid molecule according to any one of claims 1 to 7.
- 15 14. The viral vector according to claim 13 wherein said vector is selected from the group consisting of herpes simplex viral vectors, adenovirus-associated viral vectors and retroviral vectors.
 - 15. A host cell carrying a vector according to any one of claims 8 to 14.
- 16. The host cell according to claim 15 wherein said cell is selected from the group consisting of a human cell, dog cell, monkey cell, rat cell and mouse cell.
 - 17. An isolated protein, comprising a TGF-beta binding-protein encoded by the nucleic acid molecule according to any one of claims 1 to 7.
- 25 An antibody which specifically binds to the protein according to claim 17.

- 19. The antibody according to claim 18 wherein said antibody is a monoclonal antibody.
- 20. The antibody according to claim 19 wherein said monoclonal antibody is a murine or human antibody.
- 5 21. The antibody according to claim 18 wherein said antibody is selected from the group consisting of F(ab')₂, F(ab)₂, Fab', Fab, and Fv.
 - 22. A hybridoma which produces an antibody according to claim 19.
- 23. A fusion protein, comprising a first polypeptide segment comprising a TGF-beta binding-protein encoded by the nucleic acid molecule according to any one of claims 1 to 7, or a portion thereof of at least 10 amino acids in length, and a second polypeptide segment comprising a non-TGF-beta binding-protein.
 - 24. The fusion protein according to claim 23 wherein said first polypeptide segment is at least 20 amino acids in length.
- 25. The fusion protein according to claim 23 wherein said first polypeptide segment is at least 50 amino acids in length.
 - 26. The fusion protein according to claim 23 wherein said second polypeptide comprises multiple anionic amino acid residues.
- 27. An isolated oligonucleotide which hybridizes to a nucleic acid molecule according to Sequence ID NOs. 1, 3, 5, 7, 9, 11, 13, or 15, or the complement thereto, under conditions of high stringency.
 - 28. The isolated oligonucleotide according to claim 27 wherein said oligonucleotide is at least 20 nucleotides in length.
 - 29. The isolated oligonucleotide according to claim 27 wherein said oligonucleotide is at least 30 nucleotides in length.
- The isolated oligonucleotide according to claim 27 wherein said

oligonucleotide is at least 50 nucleotides in length.

- 31. The isolated oligonucleotide according to claim 27 wherein said oligonucleotide is between 50 to 100 nucleotides in length.
- 32. A pair of primers which specifically amplifies all or a portion of a nucleic acid molecule according to any one of claims 1 to 7.
 - 33. A ribozyme which cleaves RNA encoding a protein according to claim 17.
 - 34. The ribozyme according to claim 33 wherein said protein comprises the protein of Sequence ID NO. 2.
- 10 35. The ribozyme according to claim 33 wherein said protein comprises the protein of Sequence ID NO. 6.
 - 36. The ribozyme according to claim 33 wherein said RNA encodes a protein comprising the protein of Sequence ID NO. 10.
- The ribozyme according to claim 33 wherein said RNA encodes a protein comprising the protein of Sequence ID NO. 12.
 - 38. The ribozyme according to claim 33 wherein said RNA encodes a protein comprising the protein of Sequence ID NO. 14.
 - 39. The ribozyme according to claim 33 wherein said RNA encodes a protein comprising the protein of Sequence ID NO. 16.
- 20 40. The ribozyme according to claim 33 wherein said ribozyme is composed of ribonucleic acids.
 - 41. The ribozyme according to claim 40 wherein one or more of said ribonucleic acids are 2'-O-methyl ribonucleic acids.
 - 42. The ribozyme according to claim 33 wherein said ribozyme is

composed of a mixture of deoxyribonucleic acids and ribonucleic acids.

- 43. The ribozyme according to claim 33 wherein said ribozyme is composed of nucleic acids having phosphothioate linkages.
- 44. A nucleic acid molecule comprising a nucleic acid sequence which encodes a ribozyme according to claim 33.
 - 45. The nucleic acid molecule of claim 44, wherein the nucleic acid is DNA or cDNA.
 - 46. The nucleic acid molecule of claim 44, under the control of a promoter to transcribe the nucleic acid.
- 10 47. A host cell comprising the ribozyme of claim 33.
 - 48. A vector, comprising the nucleic acid molecule of claim 44.
 - 49. The vector of claim 54, wherein the vector is a plasmid, a virus, retrotransposon or a cosmid.
- 50. The vector of claim 49 wherein said virus is selected from the group consisting of retroviruses, adenoviruses, and adeno-associated viruses.
 - 51. A host cell containing the vector according to any one of claims 48 to 50.
 - 52. The host cell according to claim 51 wherein said host cell is stably transformed with said vector.
- The host cell according to claim 51 wherein the host cell is a human cell.
 - 54. A method for producing a ribozyme, comprising providing DNA encoding the ribozyme according to claim 33 under the transcriptional control of a promoter, and transcribing the DNA to produce the ribozyme.

- 55. The method of claim 54 wherein the ribozyme is produced in vitro.
- 56. The method of claim 54, further comprising purifying the ribozyme.
- 5 57. A method for increasing bone mineralization, comprising introducing into a warm-blooded animal an effective amount of the ribozyme according to any one of claims 33 to 43.
- 58. A method of increasing bone mineralization, comprising introducing into a patient an effective amount of the nucleic acid molecule of claim 44, under conditions favoring transcription of the nucleic acid molecule to produce a ribozyme.
 - 59. A pharmaceutical composition, comprising the ribozyme according to any one of claims 33 to 43, and a pharmaceutically acceptable carrier or diluent.
- 15 60. A pair of primers capable of specifically amplifying all or a portion of a nucleic acid molecule according to any one claims 1 to 7.
- 61. A method for detecting a nucleic acid molecule which encodes a TGF-beta binding protein, comprising incubating an oligonucleotide according to any one of claims 27 to 31 under conditions of high stringency, and detecting hybridization of said oligonucleotide.
 - 62. The method according to claim 61 wherein said oligonucleotide is labeled.
 - 63. The method according to claim 61 wherein said oligonucleotide is bound to a solid support.
- 25 64. A method for detecting a TGF-beta binding protein, comprising incubating an antibody according to any one of claims 18 to 21 under conditions and for a time sufficient to permit said antibody to bind to a TGF-beta binding protein, and

detecting said binding.

- 65. The method according to claim 64 wherein said antibody is bound to a solid support.
- 66. The method according to claim 64 wherein said antibody is labeled.
 - 67. The method according to claim 66 wherein said antibody is labeled with a marker selected from the group consisting of enzymes. fluorescent proteins, and radioisotopes.
- 68. A transgenic animal whose germ cells and somatic cells contain a nucleic acid molecule encoding a TGF-beta binding-protein according to claim 1 which is operably linked to a promoter effective for the expression of said gene, said gene being introduced into said animal, or an ancestor of said animal, at an embryonic stage, with the proviso that said animal is not a human.
- 69. The transgenic animal according to claim 68 wherein TGF-beta binding-protein is expressed from a vector according to any one of claims 8 to 10.
 - 70. A transgenic knockout animal, comprising an animal whose germ cells and somatic cells comprise a disruption of at least one allele of an endogenous nucleic acid molecule which hybridizes to the nucleic acid molecule according to claim 1, wherein said disruption prevents transcription of messenger RNA from said allele as compared to an animal without said disruption, with the proviso that said animal is not a human.
 - 71. The transgenic animal according to claim 70 wherein said disruption is a nucleic acid deletion, substitution, or, insertion.
- 72. The transgenic animal according to claim 68 or 70 wherein the animal is selected from the group consisting of a mouse, a rat and a dog.

- 73. A method for determining whether a candidate molecule is capable of increasing bone mineral content, comprising:
- (a) mixing one or more candidate molecules with TGF-beta-binding-protein encoded by the nucleic acid molecule according to any one of claims 1 to 7 and a selected member of the TGF-beta family of proteins;
- (b) determining whether the candidate molecule alters the signaling of the TGF-beta family member, or alters the binding of the TGF-beta binding-protein to the TGF-beta family member.
- 74. The method according to claim 73 wherein said member of the TGF-beta family of proteins is BMP6.
 - 75. A method for determining whether a candidate molecule is capable of increasing bone mineral content, comprising: determining whether a candidate molecule inhibits the binding of TGF-beta binding-protein to bone, or an analogue thereof.
- The method according to claim 75 wherein said analogue of bone is hydroxyapatite.
 - 77. A kit for detection of TGF-beta binding-protein gene expression, comprising a container that comprises a nucleic acid molecule, wherein said nucleic acid molecule is selected from the group consisting of (a) a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO: 1, 5, 7, 9, 11, 13, or 15, (b) a nucleic acid molecule comprising the complement of the nucleotide sequence of (a), (c) a nucleic acid molecule that is a fragment of (a) or (b) of at least 20 nucleotides in length.
- 78. A kit for detection of TGF-beta binding-protein, comprising a container that comprises an antibody according to any one of claims 18 to 21.
- 79. An antisense oligonucleotide, comprising a nucleic acid molecule which hybridizes to a nucleic acid molecule according to Sequence ID NOs. 1, 3, 5, 7, 9, 11, 13, or 15, or the complement thereto, and wherein said oligonucleotide inhibits the expression of TGF-beta binding protein according to

claim 17.

- The oligonucleotide according to claim 79 wherein said oligonucleotide is 15 nucleotides in length.
- The oligonucleotide according to claim 79 wherein said oligonucleotide is 20 nucleotides in length.
 - 82. The oligonucleotide according to claim 79 wherein said oligonucleotide is 50 nucleotides in length.
 - 83. The oligonucleotide according to claim 79, wherein said oligonucleotide is comprised of one or more nucleic acid analogs.
- The oligonucleotide according to claim 79, wherein said oligonucleotide is comprised of one or more ribonucleic acids.
 - 85. The oligonucleotide according to claim 79, wherein said oligonucleotide is comprised of one or more deoxyribonucleic acids.
- 86. The oligonucleotide according to claim 79 wherein said oligonucleotide sequence comrpises one or more modified covalent linkages.
- 87. The oligonucleotide according to claim 86 wherein said modified covalent linkage is selected from the group consisting of a phosphorothioate linkage, a phosphotriester linkage, a methyl phosphonate linkage, a methylene(methylimino) linkage, a morpholino linkage, an amide linkage, a polyamide linkage, a short chain alkyl intersugar linkage, a cycloalkyl intersugar linkage, a short chain heteroatomic intersugar linkage and a heterocyclic intersugar linkage.

Common Cysteine Backbone

i human-gremlin.pr	0			50	
numan-cerberus.pro human-dan.pro human-beer.pro	MHELEFQLL	V LLPLGKTTRI	QOGRONQSSL	SPVLLPRNQR	ELPTGNHEEA
human-gremlin.bro numan-cerberus.pro human-dan.pro human-beer.pro	EEKPDLFVAV	/ PHLVAT.SPA	SRTAYTVGAL GEGGROREKM	LLLLGTLLPA LSRFGRFWKK MQLPLA	100 AEGKKKGSQG PEREMHPSRD —————— LCLVCLLVHT
human-gremlin.pro human-cerberus.pro human-dan.pro human-beer.pro	SDSEPFPPGT	QSLIQPID.G	MKMEKSPLRE	GOGRGTAMPG EAKKFWHHFM MLRVLVGAVL PPELENNKTM	FRKTPASQGV PAMLLAAPPP
human-gremlin.pro human-cerberus.pro human-dan.pro human-beer.pro	ILPIKSHEVH INKLALFPDK	WETCRTVPFS SAWCEAKNIT	OTITHEGCEK QIVGHSGCEA	RTIINRF.CY (VVVQNNL.CF (KSIQNRA.CL (AKPVTELVCS (GKCGSVHFP. GOCFSYSVPN
human-gremlin.pro human-cerberus.pro human-gan.pro human-beer.pro	GAAQHSHT TFPQSTESLV	SCSHCLP. HCDSCMP.	AKFTTMHLPL N AQSMWEIVTL 1	NCPELQPPTK K NCTELSSVIK V ECPGHEEVPR V LCPGGEAPRA R	VMLVEE 'DKLVEKILH
human-gremlin.pro human-cerberus.pro human-dan.pro human-beer.pro	COCKVKTEHE (CSCOACGKEP S	DGHILHAGSQ (SHEGLSVYVQ (SFIPGVSA~ ~ GEDGPGSQPG T	НРНРНРНРН Р	GGOTPEPED
numan-gremiin.pro numan-cerberus.pro human-dan.pro numan-beer.pro	PPGAPHTEEE (Fig	r. 1

Human Beer Gene Expression by RT-PCR

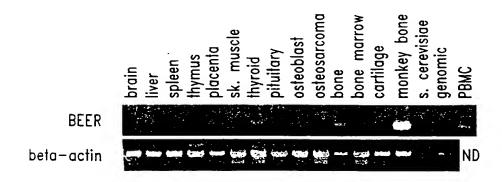
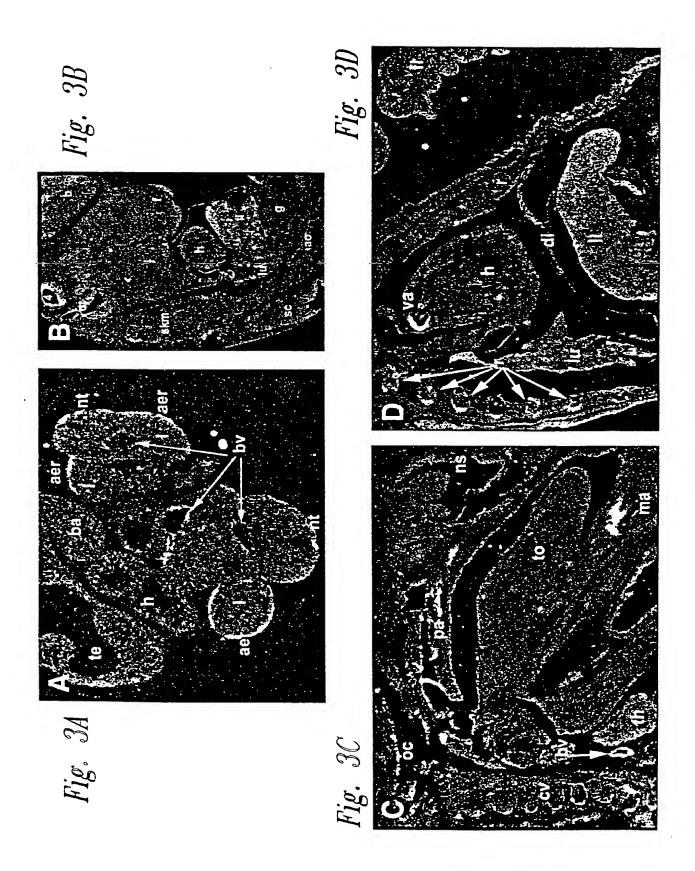


Fig. 2



		nti er		nti an	Anti Gremlin		
BEER Protein (75 ng/lane)	1:5000	1:10,000	1:1000	1:5000	1:1000	1:5000	

Fig. 4A

Anti Anti Anti Dan Beer Gremlion

Dan Protein (75 ng/lane)

Anti Anti Anti Gremlion

October Gremlion

34 kD ---

20 kD

Fig. 4C



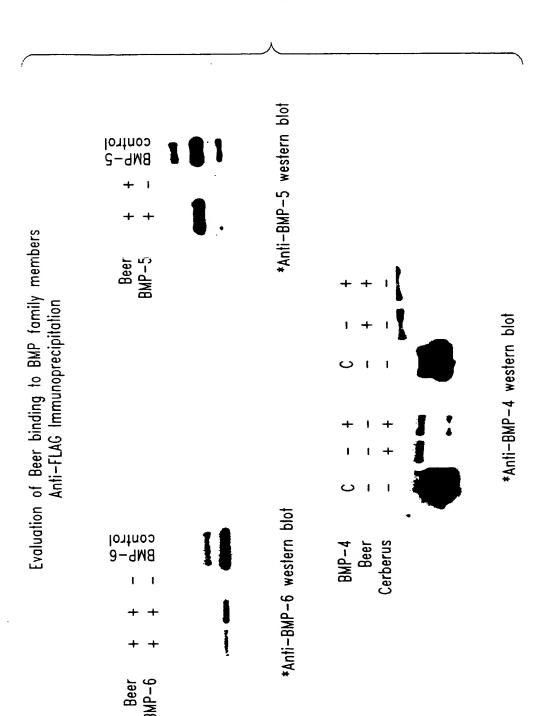


Fig. 5

BMP-5/Beer Dissociation Constant Characterization

.75 1.5 7.5 15 30 60 120 nM BMP-5



*Anti-FLAG immunoprecipitation *Anti-BMP-5 western blot

Ionic Disruption of BMP-5/Beer Binding



*Anti-FLAG immunoprecipitation *Anti-BMP-5 western blot

Fig. 6

SEQUENCE LISTING

	<110> Bru	nkow, Mary E.				
5	Gal	as, David J.				
	Kov	acevich, Brian				
	Mul	ligan, John T.				
	Pae	oer, Bryan W.				
	Van	Ness, Jeffrey				
10	Win)	ler, David G.				
	<120> COM	OSITIONS AND MET	THODS FOR IN	CREASING		
	BONE MIN	ERALIZATION				
15						
	<130> 2400	83.508				
	<140> US					
	<141> 1999	-11-24				
20						
	<160> 41					
	·					
	<170> Fast	SEQ for Windows	Version 3.0			
n e						
25	<210> 1					
	<211> 2301					
	<212> DNA					
	<213> Homo	sapier.				
30	400					
	<400> 1					
	agageetgtg ctact					60
	tggccctgtg tctcg					120
	ggtggcaggc gttca					180
35	agcctccacc ggagc					240
, ,	ctccccacca cccct	itgag accaaagacg	tgtccgagta	cagctgccgc	gagetgeact	300
	tcacccgcta cgtga	cgat gggccgtgcc	gcagcgccaa	gccggtcacc	gagctggtgt	360

	gctccggcca	gtgcggcccg	gagagaatga	tgcccaacgc	categgeege	ggcaagtggt	420
	ggcgacctag	tgggcccgac	ttccgctgca	teccegaceg	ctaccgcgcg	, cagegegege	480
	agctgctgtg	tcccggtggt	gaggcgccgc	gcgcgcgcaa	ggtgcgcctg	gtggcctcgt	540
	gcaagtgcaa	gcgcctcacc	cgcttccaca	accagtcgga	gctcaaggac	ttegggaeeg	600
5	aggccgctcg	gccgcagaag	ggccggaagc	cgcggccccg	cgcccggago	gccaaagcca	660
	accaggccga	gctggagaac	gcctactaga	gcccgcccgc	gaccatacca	accggcgggc	720
	gccccggccc	tgaacccgcg	ccccacattt	ctgtcctctg	cgcgtggttt	gattgtttat	780
	atttcattgt	aaatgcctgc	aacccagggc	agggggctga	gaccttccag	gccctgagga	840
	atcccgggcg	ccggcaaggc	ccccctcage	ccgccagctg	aggggtccca	cggggcaggg	900
10	gagggaattg	agagtcacag	acactgagcc	acgcagcccc	gcctctgggg	ccgcctacct	960
	ttgctggtcc	cacttcagag	gaggcagaaa	tggaagcatt	ttcaccgccc	tggggtttta	1020
	agggagcggt	gtgggagtgg	gaaagtccag	ggactggtta	agaaagttgg	ataagattcc	1080
	cccttgcacc	tcgctgccca	tcagaaagcc	tgaggcgtgc	ccagagcaca	agactggggg	1140
	caactgtaga	tgtggtttct	agtcctggct	ctgccactaa	cttgctgtgt	aaccttgaac	1200
15	tacacaattc	tccttcggga	cctcaatttc	cactttgtaa	aatgagggtg	gaggtgggaa	1260
	taggatctcg	aggagactat	tggcatatga	ttccaaggac	tccagtgcct	tttgaatggg	1320
	cagaggtgag	agagagagag	agaaagagag	agaatgaatg	cagttgcatt	gattcagtgc	1380
	caaggtcact	tccagaattc	agagttgtga	tgctctcttc	tgacagccaa	agatgaaaaa	1440
	caaacagaaa	aaaaaagta	aagagtctat	ttatggctga	catatttacg	gctgacaaac	1500
20	tcctggaaga	agctatgctg	cttcccagcc	tggcttcccc	ggatgtttgg	ctacctccac	1560
	ccctccatct	caaagaaata	acatcatcca	ttggggtaga	aaaggagagg	gtccgagggt	1620
	ggtgggaggg	atagaaatca	catccgcccc	aacttcccaa	agagcagcat	ccctcccccg	1680
	acccatagcc	atgttttaaa	gtcaccttcc	gaagagaagt	gaaaggttca	aggacactgg	1740
	ccttgcaggc	ccgagggagc	agccatcaca	aactcacaga	ccagcacatc	ccttttgaga	1800
25	caccgccttc	tgcccaccac	tcacggacac	atttctgcct	agaaaacagc	ttcttactgc	1860
	tcttacatgt	gatggcatat	cttacactaa	aagaatatta	ttgggggaaa	aactacaagt	1920
	gctgtacata	tgctgagaaa	ctgcagagca	taatagctgc	cacccaaaaa	tctttttgaa	1980
	aatcatttcc	agacaacctc	ttactttctg	tgtagttttt	aattgttaaa	aaaaaaagt	2040
	tttaaacaga	agcacatgac	atatgaaagc	ctgcaggact	ggtcgttttt	ttggcaattc	2100
30	ttccacgtgg	gacttgtcca	caagaatgaa	agtagtggtt	tttaaagagt	taagttacat	2160
				aaagtttttc			2220
				ttccagagtc			2280
		atgaccgaaa					2301

35 <210> 2

<211> 213

<212> PRT <213> Homo sapien

<400> 2

5	Met	Gln	Leu	Pro	Leu	Ala	Leu	Cys	Leu	ı Val	. Cys	: Leu	Leu	Val	His	Thr
	1				5					10					15	
	Ala	Phe	Arg	Val	Val	Glu	Gly	Gln	Gly	Trp	Gln	Ala	Phe	Lys	Asr	Asp
				20					25					30		·
	Ala	Thr	Glu	Ile	Ile	Pro	Glu	Leu	Gly	Glu	Tyr	Pro	Glu	Pro	Pro	Pro
10			35					40					45			
	Glu	Leu	Glu	Asn	Asn	Lys	Thr	Met	Asn	Arg	Ala	Glu	Asn	Gly	Gly	Arg
		50					55					60				
	Pro	Pro	His	His	Pro	Phe	Glu	Thr	Lys	Asp	Val	Ser	Glu	Tyr	Ser	Cys
	65					70					75					80
15	Arg	Glu	Leu	His	Phe	Thr	Arg	Tyr	Val	Thr	Asp	Gly	Pro	Cys	Arg	Ser
					85					90					95	
	Ala	Lys	Pro	Val	Thr	Glu	Leu	Val	Cys	Ser	Gly	Gln	Cys	Gly	Pro	Ala
				100					105					110		
	Arg	Leu	Leu	Pro	Asn	Ala	Ile	Gly	Arg	Gly	Lys	Trp	Trp	Arg	Pro	Ser
20			115					120					125			
	Gly	Pro	Asp	Phe	Arg	Суѕ	Ile	Pro	Asp	Arg	Tyr	Arg	Ala	Gln	Arg	Val
		130					135					140				
		Leu	Leu	Cys	Pro	Gly	Gly	Glu	Ala	Pro	Arg	Ala	Arg	Lys	Val	Arg
	145					150					155					160
25	Leu	Val	Ala	Ser	Cys	Lys	Cys	Lys	Arg	Leu	Thr	Arg	Phe	His	Asn	Gln
					165					170					175	
	Ser	Glu	Leu	Lys	Asp	Phe	Gly	Thr	Glu	Ala	Ala	Arg	Pro	Gln	Lys	Gly
				180					185					190		
•	Arg	Lys		Arg	Pro	Arg	Ala	Arg	Ser	Ala	Lys	Ala	Asn	Gln	Ala	Glu
30			195					200					205			
	Leu	Glu	Asn	Ala	Tyr											
		210														

35

<210> 3

<211> 2301 <212> DNA

<213> Homo sapien

<400> 3

	agagcctgtg	ctactggaag	gtggcgtgcc	ctcctctggc	tggtaccatg	cagcicccac	60
5	tggccctgtg	totogtotgo	ctgctggtac	acacageett	ccgtgtagtg	gagggctagg	120
	ggtggcaggc	gttcaagaat	gatgccacgg	aaatcatccc	cgagetegga	gagtaccccg	180
						ggagggcggc	240
	ctccccacca	cccctttgag	accaaagacg	tgtccgagta	cagctgccgc	gagctgcact	300
						gagctggtgt	360
10						ggcaagtggt	420
						cagcgcgtgc	480
		tcccggtggt					540
	gcaagtgcaa	gcgcctcacc	cgcttccaca	accagtcgga	gctcaaggac	ttcgggaccg	600
	aggccgctcg	gccgcagaag	ggccggaagc	cgcggccccg	cgcccggagc	gccaaagcca	660
15	accaggccga	gctggagaac	gcctactaga	gcccgcccgc	gcccctcccc	accggcgggc	720
	gccccggccc	tgaacccgcg	ccccacattt	ctgtcctctg	cgcgtggttt	gattgtttat	780
	atttcattgt	aaatgcctgc	aacccagggc	agggggctga	gaccttccag	gccctgagga	840
	atcccgggcg	ccggcaaggc	cccctcagc	ccgccagctg	aggggtccca	cggggcaggg	900
	gagggaattg	agagtcacag	acactgagcc	acgcagcccc	gcctctgggg	ccgcctacct	960
20	ttgctggtcc	cacttcagag	gaggcagaaa	tggaagcatt	ttcaccgccc	tggggtttta	1020
	agggagcggt	gtgggagtgg	gaaagtccag	ggactggtta	agaaagttgg	ataagattcc	1080
	cccttgcacc	tegetgeeca	tcagaaagcc	tgaggcgtgc	ccagagcaca	agactggggg	1140
	caactgtaga	tgtggtttct	agtcctggct	ctgccactaa	cttgctgtgt	aaccttgaac	1200
	tacacaattc	tccttcggga	cctcaatttc	cactttgtaa	aatgagggtg	gaggtgggaa	1260
25	taggatctcg	aggagactat	tggcatatga	ttccaaggac	tccagtgcct	tttgaatggg	1320
	cagaggtgag	agagagagag	agaaagagag	agaatgaatg	cagttgcatt	gattcagtgc	1380
	caaggtcact	tccagaattc	agagttgtga	tgctctcttc	tgacagccaa	agatgaaaaa	1440
	caaacagaaa	aaaaaagta	aagagtctat	ttatggctga	catatttacg	gctgacaaac	1500
	tcctggaaga	agctatgctg	cttcccagcc	tggcttcccc	ggatgtttgg	ctacctccac	1560
30	ccctccatct	caaagaaata	acatcatcca	ttggggtaga	aaaggagagg	gtccgagggt	1620
	ggtgggaggg	atagaaatca	cateegeeee	aacttcccaa	agagcagcat	ccctcccccg	1680
	acccatagcc	atgttttaaa	gtcaccttcc	gaagagaagt	gaaaggttca	aggacactgg	1740
	ccttgcaggc	ccgagggagc	agccatcaca	aactcacaga	ccagcacatc	ccttttgaga	1800
		tgcccaccac					1860
35		gatggcatat					1920
		tgctgagaaa					1980

	aatcatttoo agacaacoto ttactttotg tgtagttttt aattgttaaa aaaaaaaagt	2040
	tttaaacaga agcacatgac atatgaaagc cigcaggact ggtcgttttt tiggcaatic	2100
	ttccacgtgg gacttgtcca caagaatgaa agtagtggtt tttaaagagt taagttacat	2160
	atttattttc tcacttaagt tatttatgca aaagtttttc ttgtagagaa tgacaatgtt	2220
5	aatattgctt tatgaattaa cagtctgttc ttccagagtc cagagacatt gttaataaag	2280
	acaatgaatc atgaccgaaa g	2301
		2001
	<210> 4	
	<211> 23	
10	<212> PRT	
	<213> Homo sapien	
	<400> 4	
	Met Gln Leu Pro Leu Ala Leu Cys Leu Val Cys Leu Leu Val His Thr	
15	1 5 10 15	
	Ala Phe Arg Val Val Glu Gly	
	20	
	<210> 5	
20	<211> 2301	
	<212> DNA	
	<213> Homo sapien	
25	<400> 5	
25	agageetgtg ctactggaag gtggegtgee etectetgge tggtaceatg cageteecae	60
	tggccctgtg tctcatctgc ctgctggtac acacagcctt ccgtgtagtg gagggccagg	120
	ggtggcaggc gttcaagaat gatgccacgg aaatcatccg cgagctcgga gagtaccccg	180
	agcetecace ggagetggag aacaacaaga ecatgaaceg ggeggagaac ggagggegge	240
30	ctccccacca cccctttgag accaaagacg tgtccgagta cagctgccgc gagctgcact	300
3()	tcaccegeta egtgacegat gggeegtgee geagegeeaa geeggteace gagetggtgt	360
	geteeggeea gtgeggeeeg gegegeetge tgeecaaege categgeege ggeaagtggt	420
	ggcgacctag tgggcccgac ticcgctgca tccccgaccg ctaccgcgcg cagcgcgtgc	480
	agetgetgtg teceggtggt gaggegeege gegegegeaa ggtgegeetg gtggeetegt	540
35	gcaagtgcaa gcgcctcacc cgcttccaca accagtcgga gctcaaggac ttcgggaccg	600
رر	aggccgctcg gccgcagaag ggccggaagc cgcggccccg cgcccggagc gccaaagcca	660
	accaggeega getggagaae geetaetaga geeegeeege geeeeteeee aceggeggge	720

	gccccggccc	tgaacccgcg	ccccacattt	ctgtcctctg	cgcgtggttt	gattgtttat	780
	atttcattgt	aaatgcctgc	aacccagggc	agggggctga	gaccttccag	gccctgagga	840
	atcccgggcg	ccggcaaggc	ccccctcage	ccgccagctg	aggggtccca	cggggcaggg	900
	gagggaattg	agagtcacag	acactgagcc	acgcagcccc	gcctctgggg	cogoctacct	960
5	ttgctggtcc	cacttcagag	gaggcagaaa	tggaagcatt	ttcaccgccc	tggggtttta	1020
	agggagcggt	grgggagrgg	gaaagtccag	ggactggtta	agaaagttgg	ataagattcc	1080
	cccttgcacc	tcgctgccca	tcagaaagcc	tgaggcgtgc	ccagagcaca	agactggggg	1140
	caactgtaga	tgtggtttct	agtcctggct	ctgccactaa	cttgctgtgt	aaccttgaac	1200
	tacacaattc	tccttcggga	cctcaatttc	cactttgtaa	aatgagggtg	gaggtgggaa	1260
10	taggateteg	aggagactat	tggcatatga	ttccaaggac	tccagtgcct	tttgaatggg	1320
	cagaggtgag	agagagagag	agaaagagag	agaatgaatg	cagttgcatt	gattcagtgc	1380
	caaggtcact	tccagaattc	agagttgtga	tgctctctc	tgacagccaa	agatgaaaaa	1440
	caaacagaaa	aaaaaaagta	aagagtctat	ttatggctga	catatttacg	gctgacaaac	1500
	tcctggaaga	agctatgctg	cttcccagcc	tggcttcccc	ggatgtttgg	ctacctccac	1560
15			acatcatcca				1620
			cateegeeee				1680
			gtcaccttcc				1740
	ccttgcaggc	ccgagggagc	agccatcaca	aactcacaga	ccagcacatc	ccttttgaga	1800
			tcacggacac				1860
20			cttacactaa				1920
			ctgcagagca				1980
			ttactttctg				2040
			atatgaaagc				2100
			caagaatgaa				2160
25			tatttatgca				2220
			cagtctgttc	ttccagagtc	cagagacatt	gttaataaag	2280
	acaatgaatc	atgaccgaaa	g				2301

<210> 6

30 <211> 213

<212> PRT

<213> Homo sapien

<400> 6

Met Gln Leu Pro Leu Ala Leu Cys Leu Ile Cys Leu Leu Val His Thr

1 5 10 15

Ala Thr Glu Ile Ile Arg Glu Leu Gly Glu Tyr Pro Glu Pro Pro Pro Pro 35		Ala	Phe	Arg		Val	Glu	Gly	Gln	Gly	Trp	Gln	Ala	Phe	Lys	As:	n Asp	
5 Glu Leu Glu Asn Asn Lys Thr Met Asn Arg Ala Glu Asn Gly Gly Arg 50					20													
5 Glu Leu Glu Asn Asn Lys Thr Met Asn Arg Ala Clu Asn Gly Gly Arg 50 55 60 Pro Pro His His Pro Phe Glu Thr Lys Asp Val Ser Glu Tyr Ser Cys 65 70 75 80 Arg Glu Leu His Phe Thr Arg Tyr Val Thr Asp Gly Pro Cys Arg Ser 10 85 90 95 Ala Lys Pro Val Thr Glu Leu Val Cys Ser Gly Gln Cys Gly Pro Ala 100 105 110 Arg Leu Leu Pro Asn Ala Ile Gly Arg Gly Lys Trp Trp Arg Pro Ser 115 120 125 15 Gly Pro Asp Phe Arg Cys Ile Pro Asp Arg Tyr Arg Ala Gln Arg Val 130 135 140 Gln Leu Leu Cys Pro Gly Gly Glu Ala Pro Arg Ala Arg Lys Val Arg 145 150 160 Leu Val Ala Ser Cys Lys Cys Lys Arg Leu Thr Arg Phe His Asn Gln 186 187 189 190 Arg Lys Pro Arg Pro Arg Ala Arg Ser Ala Lys Ala Asn Gln Ala Glu 195 200 205 25 Leu Glu Asn Ala Tyr 210 4210 7 4211 2301 4400 7 agagcctgtg ctactggaag gtggcgtgcc ctcctctggc tggtaccatg cagctccac 60 tggccctgtg tctcctctgc ctgctggtac acacagcctt ccgtgatgg gagggccagg		Ala	Thr		Ile	Ile	Arg	Glu	Leu	Gly	Glu	Tyr	Pro	Glu	Pro	Pro	Pro	
So																		
Pro	5	Glu	Leu	Glu	Asn	Asn	Lys	Thr	Met	Asn	Arg	Ala	Glu	Asn	Gly	Gly	Arg	
Arg Glu Leu His Phe Thr Arg Tyr Val Thr Asp Gly Pro Cys Arg Ser																		
Arg Glu Leu His Phe Thr Arg Tyr Val Thr Asp Gly Pro Cys Arg Ser 10 85 90 95 Ala Lys Pro Val Thr Glu Leu Val Cys Ser Gly Gln Cys Gly Pro Ala 100 105 110 Arg Leu Leu Pro Asn Ala Ile Gly Arg Gly Lys Trp Trp Arg Pro Ser 115 120 121 130 135 140 Gln Leu Leu Cys Pro Gly Gly Glu Ala Pro Arg Ala Gln Arg Val 130 Gln Leu Leu Cys Pro Gly Gly Glu Ala Pro Arg Ala Arg Lys Val Arg 145 150 Ser Glu Leu Lys Asp Phe Gly Thr Glu Ala Ala Arg Pro Gln Lys Gly 185 Ser Glu Leu Lys Asp Pro Arg Ala Arg Ser Ala Lys Ala Arg Gln Lys Gly 180 Arg Lys Pro Arg Pro Arg Ala Arg Ser Ala Lys Ala Arg Gln Ala Glu 195 200 205 25 Leu Glu Asn Ala Tyr 210 210 221 220 225 Leu Glu Asn Ala Tyr 220 235 Leu Glu Asn Ala Tyr 2210 240> 7 2213> Homo sapien		Pro	Pro	His	His	Pro	Phe	Glu	Thr	Lys	qzA	Val	Ser	Glu	Tyr	Ser	Cys	
10							_											
Ala Lys Pro Val Thr Glu Leu Val Cys Ser Gly Gln Cys Gly Pro Ala 100 1		Arg	Glu	Leu	His	Phe	Thr	Arg	Tyr	Val	Thr	Asp	Gly	Pro	Cys	Arg	Ser	
Arg Leu Leu Pro Asn Ala Ile Gly Arg Gly Lys Trp Trp Arg Pro Ser 115	10																	
Arg Leu Leu Pro Asn Ala Ile Gly Arg Gly Lys Trp Trp Arg Pro Ser 115		Ala	Lys	Pro	Val	Thr	Glu	Leu	Val	Cys	Ser	Gly	Gln	Cys	Gly	Pro	Ala	
115 120 125 128 128 128 128 128 128 128 130																		
115 120 125 128 128 128 128 128 128 128 130		Arg	Leu	Leu	Pro	Asn	Ala	Ile	Gly	Arg	Gly	Lys	Trp	Trp	Arg	Pro	Ser	
130				115					120					125				
Gln Leu Leu Cys Pro Gly Gly Glu Ala Pro Arg Ala Arg Lys Val Arg 145	15	Gly	Pro	Asp	Phe	Arg	Cys	Ile	Pro	qzA	Arg	Tyr	Arg	Ala	Gln	Arg	Val	
145																		
Leu Val Ala Ser Cys Lys Cys Lys Arg Leu Thr Arg Phe His Asn Gln 20 Ser Glu Leu Lys Asp Phe Gly Thr Glu Ala Ala Arg Pro Gln Lys Gly 180 Arg Lys Pro Arg Pro Arg Ala Arg Ser Ala Lys Ala Asn Gln Ala Glu 195 200 Leu Glu Asn Ala Tyr 210 <pre></pre>		Gln	Leu	Leu	Cys	Pro	Gly	Gly	Glu	Ala	Pro	Arg	Ala	Arg	Lys	Val	Arg	
20																		
Ser Glu Leu Lys Asp Phe Gly Thr Glu Ala Ala Arg Pro Gln Lys Gly 180		Leu	Val	Ala	Ser	Cys	Lys	Cys	Lys	Arg	Leu	Thr	Arg	Phe	His	Asn	Gln	
Arg Lys Pro Arg Pro Arg Ala Arg Ser Ala Lys Ala Asn Gln Ala Glu	20																	
Arg Lys Pro Arg Pro Arg Ala Arg Ser Ala Lys Ala Asn Gln Ala Glu 195 200 205 Leu Glu Asn Ala Tyr 210		Ser	Glu	Leu	Lys	Asp	Phe	Gly	Thr	Glu	Ala	Ala	Arg	Pro	Gln	Lys	Gly	
Leu Glu Asn Ala Tyr 210 <pre></pre>																		
Leu Glu Asn Ala Tyr 210 <pre></pre>		Arg	Lys	Pro	Arg	Pro	Arg	Ala	Arg	Ser	Ala	Lys	Ala	Asn	Gln	Ala	Glu	
<pre></pre>									200					205				
<pre></pre>	25	Leu	Glu	Asn	Ala	Tyr												
<pre></pre>			210															
<pre></pre>																		
30 <212> DNA <213> Homo sapien <400> 7 agagectgtg ctactggaag gtggegtgee etcetetgge tggtaceatg cageteecac 60 35 tggeeetgtg tetegtetge etgetggtae acacageett cegtgtagtg gagggeeagg 120		4.																
<pre></pre>			<2	11>	2301													
<pre><400> 7 agagcctgtg ctactggaag gtggcgtgcc ctcctctggc tggtaccatg cagctcccac 60 tggccctgtg tctcgtctgc ctgctggtac acacagcctt ccgtgtagtg gagggccagg 120</pre>	30		<2	12>	DNA													
agageetgtg etaetggaag gtggegtgee eteetetgge tggtaceatg eageteecae 60 15 tggeeetgtg tetegtetge etgetggtae acaeageett eegtgtagtg gagggeeagg 120			<2	13>	Homo	sap	ien											
agageetgtg etaetggaag gtggegtgee eteetetgge tggtaceatg eageteecae 60 15 tggeeetgtg tetegtetge etgetggtae acaeageett eegtgtagtg gagggeeagg 120																		
35 tggccctgtg tctcgtctgc ctgctggtac acacagcctt ccgtgtagtg gagggccagg 120																		
35 tggccctgtg tctcgtctgc ctgctggtac acacagcctt ccgtgtagtg gagggccagg 120		agag	cctg	tg c	tact	ggaa	g gt	ggcg	tgcc	CEC	ctct	ggc t	tggta	accat	g ca	agct	ccac	60
ggtggcaggc gttcaagaat gatgccacgg aaatcatccg cgagctcgga gagtaccccg 180	35	tggc	cctg	tg t	ctcg	tctg	C Ct	gctg	gtac	aca	cage	ctt d	cgt	gtagt	g ga	aggg(ccagg	120
		ggtg	gcag	gc g	ttca	agaa	t gai	tgcc	acgg	aaaı	cato	ccg d	gago	ctcgg	ga ga	agtad	cccg	180

	2000-0020						
	agectecace	ggagetggag	g aacaacaaga	a ccatgaacc	g ggcggagaa	ggagggggg	240
	CtCCCcacca	cccctttgag	gaccaaagaco] tgtccgagt	a cagetgeeg	gagetgeact	300
	tcacccgcta	cgtgaccgat	gggccgtgcc	gcagcgcca	a googgtcac	gagetggtgt	360
	geteeggeea	gtgcggcccg	gegegeetge	tgcccaacg	c categgeeg	ggcaagtggt	420
5	ggcgacctag	rgggcccgac	ttccgctgca	teccegace	g ctaccgcgcg	g cagegegege	480
	agctgctgtg	teceggtggt	gaggcgccgc	gegegegea	a ggtgcgcctg	gtggcctcgt	540
	gcaagtgcaa	gegeeteace	cgcttccaca	accagtcgg	a gctcaaggad	ttcgggaccg	600
	aggccgctcg	gccgcagaag	ggccggaagc	cgcggccccg	g cgcccggagc	gccaaagcca	660
	accaggccga	gctggagaac	gcctactaga	gcccgcccg	gacactacaa	accggcgggc	720
10	geeeeggeee	tgaacccgcg	ccccacattt	ctgtcctctg	g cgcgtggttt	gattgtttat	780
	atttcattgt	aaatgcctgc	aacccagggc	agggggctga	gaccttccag	gccctgagga	840
	atcccgggcg	ccggcaaggc	ccccctcagc	ccgccagetg	g aggggtccca	cggggcaggg	900
	gagggaattg	agagtcacag	acactgagcc	acgcagcccc	gcctctgggg	ccgcctacct	960
	ttgctggtcc	cacttcagag	gaggcagaaa	tggaagcatt	ttcaccgccc	tggggtttta	1020
15	agggagcggt	gtgggagtgg	gaaagtccag	ggactggtta	agaaagttgg	ataagattcc	1080
	CCCTTGCacc	tcgctgccca	tcagaaagcc	tgaggcgtgc	ccagagcaca	agactggggg	1140
	caactgtaga	tgtggtttct	agtcctggct	ctgccactaa	cttgctgtgt	aaccttgaac	1200
	tacacaattc	tccttcggga	cctcaatttc	cactttgtaa	aatgagggtg	gaggtgggaa	1260
	taggateteg	aggagactat	tggcatatga	ttccaaggac	tccagtgcct	tttgaatggg	1320
20	cagaggtgag	agagagagag	agaaagagag	agaatgaatg	cagttgcatt	gattcagtgc	1380
	caaggtcact	tccagaattc	agagttgtga	tgctctcttc	tgacagccaa	agatgaaaaa	1440
	caaacagaaa	aaaaaagta	aagagtctat	ttatggctga	catatttacg	gctgacaaac	1500
	tcctggaaga	agctatgctg	cttcccagcc	tggcttcccc	ggatgtttgg	ctacctccac	1560
	ccctccatct	caaagaaata	acatcatcca	ttggggtaga	aaaggagagg	gtccgagggt	1620
25	ggtgggaggg	atagaaatca	catccgccc	aacttcccaa	agagcagcat	CCCtccccq	1680
	acccatagcc	atgttttaaa	gtcaccttcc	gaagagaagt	gaaaggttca	aggacactgg	1740
	ccttgcaggc	ccgagggagc	agccatcaca	aactcacaga	ccagcacatc	CCTTTTGaga	1800
	caccgccttc	tgcccaccac	tcacggacac	atttctgcct	agaaaacagc	ttettactor	1860
	tcttacatgt	gatggcatat	cttacactaa	aagaatatta	ttgggggaaa	aactacaagt	1920
30	gctgtacata	tgctgagaaa	ctgcagagca	taatagctgc	cacccaaaaa	tCtttttgaa	1980
	aatcatttcc	agacaacctc	ttactttctg	tgtagttttt	aattgttaaa	aaaaaaaar	2040
	tttaaacaga	agcacatgac	atatgaaagc	ctgcaggact	ggtcgttttt	ttagcaatto	2100
	ttccacgtgg	gacttgtcca	caagaatgaa	agtagtggtt	tttaaagagt	taagtracar	2160
	atttattttc	tcacttaagt	tatttatgca	aaagttttc	ttgtagagaa	geedeat	2220
35	aatattgctt	tatgaattaa	cagtctgttc	ttccagaqtc	cagagacatt (oftaataaaa	2220
	acaatgaatc	atgaccgaaa (9	J J - C	J-340466 \	uucuaay	
							2301

		<	210>	8												
		<	211>	213												
		<	212>	PRT	•											
5		<	213>	Hom	o sa	pien	1									
		<	400>	8												
	Met	Gln	Leu	Pro	Leu	Ala	Leu	Cys	Leu	Val	Cvs	Leu	Leu	. Val	His	Thr
	1				5					10	•				15	,
10	Ala	Phe	Arg	Val	Val	Glu	Gly	Gln	Glv	Tro	Gln	Ala	Pha	Lare		Asp
				20					25				- 110	30	, Hell	ASP
	Ala	Thr	Glu	Ile	Ile	Arg	Glu	Leu		Glu	Tvr	Pro	Glu		D~o	Pro
			35					40	•		-1-		45		r i O	FIO
	Glu	Leu	Glu	Asn	Asn	Lys	Thr	Met	Asn	Ara	Ala	Glu		G1v	Cl.	Arg
15		50				_	55			5		60	A3.1	GIY	GIY	Arg
	Pro	Pro	His	His	Pro	Phe	Glu	Thr	Lvs	Asn	Val		Clu	Т	C	Cys
	65					70			-70		75	Jei	GIU	ıyı	Sei	
	Arg	Glu	Leu	His	Phe	Thr	Ara	Tvr	Val	Thr		Cly	Dro	C	Arg	80
					85		_	- 2		90	лэр	Gly	PIO	Cys		ser
20	Ala	Lys	Pro	Val	Thr	Glu	Leu	Val	Cve		Glyr	C1-	C	01	95 Pro	
				100					105	361	GIY	GIII	Cys		Pro	Ala
	Arg	Leu	Leu	Pro	Asn	Ala	Tla	Gly		Clyr	T 1 10	T	m	110	Pro	_
			115					120	Arg	GIY	гÀг	Irp		Arg	Pro	Ser
	Gly	Pro		Phe	Ara	Cvs	Tlo		Λαπ	λ 	T	2	125		Arg	
25	-	130			5	0,0	135	110	Asp	Arg	ıyr		Ala	Gin	Arg	Val
	Gln		Leu	Cvs	Pro	Glv		Glu	ת א	Dwa	3	140	_		Val	
	145			-, -		150	Ory	Giu	MIG	PIO		Ala	Arg	Lys	Val	Arg
	Leu	Val	Ala	Ser	Cvs		Cvc	T 1/0	λ ~ ~	7	155					160
				001	165	БуЗ	Cys	пуѕ	Arg		Inr	Arg	Phe	His	Asn	Gln
30	Ser	Glu	T.eu	Live		Dino	C1	m b	6 3	170					175	
		014	200	180	vaħ	FILE	GIY	inr		Ala	Ala	Arg	Pro	Gln	Lys	Gly
	Ara	Lve	Pro		D	2		_	185					190		
	*** 9	IJΥS		Arg	LI.O	arg	Ala		Ser	Ala	Lys	Ala	Asn	Gln	Ala	Glu
	ī eu	Cl	195	3.1 ·	_			200					205			
35	reu		ASN	Ala	Tyr											
رد		210														

```
<210> 9
            <211> 642
            <212> DNA
            <213> Cercopithecus pygerythrus
 5
            <400> 9
     atgcagetee castggeest gtgtettgte tgcctgctgg tacacgcage cttccgtgta
                                                                              60
      grggagggcc aggggrggca ggccrrcaag aargargcca cggaaarcar ccccgagcre
                                                                             120
     ggagagtacc ccgagcctcc accggagctg gagaacaaca agaccatgaa ccgggcggag
                                                                             180
     aatggaggge ggcctcccca ccacccttt gagaccaaag acgtgtccga gtacagctgc
10
                                                                             240
     cgagagetge actteaceeg etaegtgace gatgggeegt geegeagege caageeagte
                                                                             300
     accgagttgg tgtgctccgg ccagtgcggc ccggcacgcc tgctgcccaa cgccatcggc
                                                                             360
     cgcggcaagt ggtggcgccc gagtgggccc gacttccgct gcatccccga ccgctaccgc
                                                                             420
     gegeagegtg tgeagetget gtgteeeggt ggtgeegege egegegege caaggtgege
                                                                             480
15
     ctggtggcct cgtgcaagtg caagegeete accegettee acaaceagte ggageteaag
                                                                             540
     gactteggte eegaggeege teggeegeag aagggeegga ageegeggee eegegeeegg
                                                                             600
     ggggccaaag ccaatcaggc cgagctggag aacgcctact ag
                                                                             642
           <210> 10
20
           <211> 213
           <212> PRT
           <213> Cercopithecus pygerythrus
           <400> 10
     Met Gln Leu Pro Leu Ala Leu Cys Leu Val Cys Leu Leu Val His Ala
25
                      5
                                          10
                                                              15
     Ala Phe Arg Val Val Glu Gly Gln Gly Trp Gln Ala Phe Lys Asn Asp
                 20
                                      25
     Ala Thr Glu Ile Ile Pro Glu Leu Gly Glu Tyr Pro Glu Pro Pro
30
             35
                                  40
                                                      45
     Glu Leu Glu Asn Asn Lys Thr Met Asn Arg Ala Glu Asn Gly Gly Arg
                              55
     Pro Pro His His Pro Phe Glu Thr Lys Asp Val Ser Glu Tyr Ser Cys
     65
                                              75
                                                                  80
     Arg Glu Leu His Phe Thr Arg Tyr Val Thr Asp Gly Pro Cys Arg Ser
35
                     85
                                          90
                                                              95
```

	Ald L	ys Pro	vai	Thr	Glu	Leu	Val	Cys	Ser	Gly	Gln	Cys	Gly	Pro	Ala	
			100					105					110			
	Arg L	eu Leu	ı Pro	Asn	Ala	Ile	Gly	Arg	Gly	Lys	Trp	Trp	Arg	Pro	Ser	
		115					120					125				
5	Gly Pi	ro Asp	Phe	Arg	Cys	Ile	Pro	Asp	Arg	Tyr	Arg	Ala	Gln	Arg	Val	
		30				135					140					
	Gln Le	eu Leu	Cys	Pro	Gly	Gly	Ala	Ala	Pro	Arg	Ala	Arg	Lys	Val	Arg	
	145				150					155					160	
	Leu Va	al Ala	Ser	Cys	Lys	Cys	Lys	Arg	Leu	Thr	Arg	Phe	His	Asn	Gln	
10				165					170					175		
	Ser Gl	u Leu	Lys	Asp	Phe	Gly	Pro	Glu	Ala	Ala	Arg	Pro	Gln	Lys	Gly	
			180					185					190			
	Arg Ly	s Pro	Arg	Pro	Arg	Ala	Arg	Gly	Ala	Lys	Ala	Asn	Gln	Ala	Glu	
		195					200					205				
15	Leu Gl	u Asn	Ala	Tyr												
	21	0														
		<210>														
		<211>														
20		<212>	DNA													
		<213>	Mus	musc	ulus											
		<400>														
3.5	atgcag	ccct c	acta	gccc	c gt	gcct	catc	tgc	ctac	ttg 1	tgca	cgct	gc c	ttct	gtgct	60
25	gtggagg	ggcc a	19999	tggc	a ag	CCTT	cagg	aat	gatg	cca d	caga	ggtca	at c	ccag	ggctt	120
	ggagagt	cacc c	cgag	CCTC	c tc	ctga	gaac	aac	caga	ca ı	tgaa	cggg	gc g	gaga	atgga	180
	ggcagad	cctc c	ccac	catc	C Cta	atga	cgcc	aaa	ggtgi	gt	cga	gtaca	ag c	rgcc	gcgag	240
	ctgcact	caca c	ccgc	ttcci	ga (caga	egge	cca	gccg	gca g	gcgc	aago	cc g	gtcad	ccgag	300
.	ttggtgt	get e	cggc	cagt	g cgg	gccc	gcg	cgg	tgct	gc d	caad	gcca	at c	gggcg	gcgtg	360
30	aagtggt	ggc g	cccg	aacg	g acc	ggat	ttc	cgct	gcat	cc c	ggat	cgct	a co	gege	gcag	420
	cgggtgc	agc t	gctg	tgcc	cgg	9999	gcg	gcgd	cgcg	gct c	gcgc	aagg	jt go	gtct	ggtg	480
	gcctcgt	gca a	gtgc	aagco	g cct	caco	cgc	ttco	cacaa	icc a	grcg	gago	t ca	aagga	cttc	540
	gggccgg	gaga c	cgcg	egged	gca	gaag	ggt	cgca	agco	gc g	gccc	ggcg	וכ כנ	gggg	jagcc	600
	aaagcca	acc a	ggcgg	gaget	gga	gaac	gcc	tact	agag	i						638
35																-

<210> 12

<211> 211	
<212> PRT	
<213> Mus mu	sculus

3		<	400>	12												
	Met	Gln	Pro	Ser	Leu	Ala	Pro	Cys	Leu	Ile	Cys	Leu	Leu	Val	His	Ala
	1				5					10					15	
	Ala	Phe	Cys	Ala	Val	Glu	Gly	Gln	Gly	Trp	Gln	Ala	Phe	Arg	Asn	Asp
				20					25					30		
10	Ala	Thr	Glu	Val	Ile	Pro	Gly	Leu	Gly	Glu	Tyr	Pro	Glu	Pro	Pro	Pro
			35					40					45			
	Glu	Asn	Asn	Gln	Thr	Met	Asn	Arg	Ala	Glu	Asn	Gly	Gly	Arg	Pro	Pro
		50					55					60				
	His	His	Pro	Tyr	Asp	Ala	Lys	Asp	Val	Ser	Glu	Tyr	Ser	Cys	Arg	Glu
15	65					70					75					80
	Leu	His	Tyr	Thr	Arg	Phe	Leu	Thr	qzA	Gly	Pro	Cys	Arg	Ser	Ala	Lys
					85					90					95	
	Pro	Val	Thr	Glu	Leu	Val	Cys	Ser	Gly	Gln	Cys	Gly	Pro	Ala	Arg	Leu
				100					105					110		
20	Leu	Pro	Asn	Ala	Ile	Gly	Arg	Val	Lys	Trp	Trp	Arg	Pro	Asn	Gly	Pro
			115					120					125			
	Asp		Arg	Cys	Ile	Pro	Asp	Arg	Tyr	Arg	Ala	Gln	Arg	Val	Gln	Leu
		130					135					140				
		Cys	Pro	Gly	Gly	Ala	Ala	Pro	Arg	Ser	Arg	Lys	Val	Arg	Leu	Val
25	145					150					155					160
	Ala	Ser	Cys	Lys	Cys	Lys	Arg	Leu	Thr	Arg	Phe	His	Asn	Gln	Ser	Glu
					165					170					175	
	Leu	Lys	Asp	Phe	Gly	Pro	Glu	Thr	Ala	Arg	Pro	Gln	Lys	Gly	Arg	Lys
	•			180					185					190		
30	Pro	Arg	Pro	Gly	Ala	Arg	Gly	Ala	Lys	Ala	Asn	Gln	Ala	Glu	Leu	Glu
			195					200					205			
	Asn	Ala	Tyr													

210

35 <210> 13 <211> 674 <212> DNA

<213> Rattus norvegicus

<400> 13

5	gaggaccgag	tgcccttcct	ccttctggca	ccatgcagct	ctcactagcc	ccttgccttg	60
10	cctgcctgct	tgtacatgca	gccttcgttg	ctgtggagag	ccaggggtgq	caagccttca	120
	agaatgatgc	cacagaaatc	atcccgggac	tcagagagta	cccagageet	cctcaggaac	180
	tagagaacaa	ccagaccatg	aaccgggccg	agaacggagg	cagaccccc	caccatcott	240
	atgacaccaa	agacgtgtcc	gagtacagct	gccgcgagct	gcactacacc	cacticataa	300
	ccgacggccc	gtgccgcagt	gccaagccgg	tcaccgagtt	ggtgtgctca	gaccaataca	360
	gccccgcgcg	gctgctgccc	aacgccatcg	ggcgcgtgaa	gragiage	Coaacogas	420
	ccgacttccg	ctgcatcccg	gatcgctacc	gcgcgcagcq	ggtgcagctg	ctatacaca	480
	gcggcgcggc	gccgcgctcg	cgcaaggtgc	gtctgqtqqc	Ctcqtqcaaq	tacaaacac	
	tcacccgctt	ccacaaccag	teggagetea	aggacttcgg	acctgagacc	acacaaccac	540
15	agaagggtcg	caagccgcgg	cccgcgccc	ggggagccaa	agccaaccag	acadaacaa	600
	agaacgccta				-goodaceag	acadaderaa	660
							674

<210> 14

<211> 213

20 <212> PRT

<213> Rattus norvegicus

<400> 14

Met Gln Leu Ser Leu Ala Pro Cys Leu Ala Cys Leu Leu Val His Ala 25 1 10 Ala Phe Val Ala Val Glu Ser Gln Gly Trp Gln Ala Phe Lys Asn Asp 25 Ala Thr Glu Ile Ile Pro Gly Leu Arg Glu Tyr Pro Glu Pro Pro Gln 35 40 Glu Leu Glu Asn Asn Gln Thr Met Asn Arg Ala Glu Asn Gly Gly Arg 30 60 Pro Pro His His Pro Tyr Asp Thr Lys Asp Val Ser Glu Tyr Ser Cys 70 75 Arg Glu Leu His Tyr Thr Arg Phe Val Thr Asp Gly Pro Cys Arg Ser 35 Ala Lys Pro Val Thr Glu Leu Val Cys Ser Gly Gln Cys Gly Pro Ala

	100 105 110	
	Arg Leu Leu Pro Asn Ala Ile Gly Arg Val Lys Trp Trp Arg Pro Asn	
	115 120 125	
	Gly Pro Asp Phe Arg Cys Ile Pro Asp Arg Tyr Arg Ala Gln Arg Val	
5	130 135 140	
	Gln Leu Leu Cys Pro Gly Gly Ala Ala Pro Arg Ser Arg Lys Val Arg	
	145 150 155 160	
	Leu Val Ala Ser Cys Lys Cys Lys Arg Leu Thr Arg Phe His Asn Gln	
	165 170 175	
10	Ser Glu Leu Lys Asp Phe Gly Pro Glu Thr Ala Arg Pro Gln Lys Gly	
	180 185 190	
	Arg Lys Pro Arg Pro Arg Ala Arg Gly Ala Lys Ala Asn Gln Ala Glu	
	195 200 205	
	Leu Glu Asn Ala Tyr	
15	210	
	<210> 15	
	<211> 532	
••	<212> DNA	
20	<213> Bos torus	
	<400> 15	
	agaargatge cacagaaare ateecegage tgggegagra eeeegageet etgeeagage	60
26	tgaacaacaa gaccatgaac cgggcggaga acggagggag acctccccac cacccctttg	120
25	agaccaaaga cgcctccgag tacagctgcc gggagctgca cttcacccgc tacgtgaccg	180
	atgggccgtg ccgcagcgcc aagccggtca ccgagctggt gtgctcgggc cagtgcggcc	240
	eggegegeet getgeecaac gecateggee geggeaagtg gtggegeeca agegggeeeg	300
	acticegetg cateceegae egetacegeg egeagegggt geagetgitg tgicetggeg	360
20	gegeggegee gegegege aaggtgegee tggtggeete gtgcaagtge aagegeetea	420
30	ctcgcttcca caaccagtcc gagctcaagg acttcgggcc cgaggccgcg cggccgcaaa	480
	cgggccggaa gctgcggccc cgcgcccggg gcaccaaagc cagccgggcc ga	532
	<210> 16	
35	<211> 176	
33	<212> PRT	
	<213> Bos torus	

```
<400> 15
      Asn Asp Ala Thr Glu Ile Ile Pro Glu Leu Gly Glu Tyr Pro Glu Pro
                        5
                                           10
      Leu Pro Glu Leu Asn Asn Lys Thr Met Asn Arg Ala Glu Asn Gly Gly
                  20
                                       25
      Arg Pro Pro His His Pro Phe Glu Thr Lys Asp Ala Ser Glu Tyr Ser
                                   4 C
      Cys Arg Glu Leu His Phe Thr Arg Tyr Val Thr Asp Gly Pro Cys Arg
10
          5 C
                               55
      Ser Ala Lys Pro Val Thr Glu Leu Val Cys Ser Gly Gln Cys Gly Pro
                          70
                                               75
      Ala Arg Leu Leu Pro Asn Ala Ile Gly Arg Gly Lys Trp Trp Arg Pro
                      85
      Ser Gly Pro Asp Phe Arg Cys Ile Pro Asp Arg Tyr Arg Ala Gln Arg
                  100
                                       105
      Val Gln Leu Leu Cys Pro Gly Gly Ala Ala Pro Arg Ala Arg Lys Val
      Arg Leu Val Ala Ser Cys Lys Cys Lys Arg Leu Thr Arg Phe His Asn
20
          130
                              135
                                                   140
      Gln Ser Glu Leu Lys Asp Phe Gly Pro Glu Ala Ala Arg Pro Gln Thr
                          150
                                               155
     Gly Arg Lys Leu Arg Pro Arg Ala Arg Gly Thr Lys Ala Ser Arg Ala
                      165
                                          170
25
            <210> 17
            <211> 35828
            <212> DNA
            <213> Mus musculus
30
            <220>
           <221> misc_feature
           <222> (1)...(35828)
           <223> n = A, T, C or G
35
```

<400> 17

	cgcgttttgg	tgagtagtaa	tattgcgctt	cgatgagcct	tggcgttgag	attgatacct	6 (
	ctgctgcaca	aaaggcaatc	gaccgagctg	gaccagcgca	ttcgtgacac	agtataatta	120
	gaacttattc	gcaatggagt	gtcattcatc	aaggacngcc	tgatcgcaaa	tggtgctatc	180
	cacgcagcgg	caatcgaaaa	ccctcagccg	gtgaccaata	tctacaacat	cagccttggt	240
5	atcctgcgtg	atgagccagc	gcagaacaag	gtaaccgtca	gtgccgataa	gttcaaagtt	300
	aaacctggtg	ttgataccaa	cattgaaacg	ttgatcgaaa	acgcgctgaa	aaacgctgct	360
	gaatgtgcgg	cgctggatgt	cacaaagcaa	atggcagcag	acaagaaagc	gatggatgaa	420
	ctggcttcct	atgtccgcac	ggccatcatg	atggaatgtt	tccccggtgg	tgttatctgg	480
	cagcagtgcc	gtcgatagta	tgcaattgat	aattattatc	atttgcgggt	cctttccggc	540
10	gatccgcctt	gttacggggc	ggcgacctcg	cgggttttcg	ctatttatga	aaattttccg	600
	gtttaaggcg	tttccgttct	tcttcgtcat	aacttaatgt	ttttatttaa	aataccctct	660
	gaaaagaaag	gaaacgacag	gtgctgaaag	cgagettttt	ggcctctgtc	gtttcctttc	720
	tctgtttttg	tccgtggaat	gaacaatgga	agtcaacaaa	aagcagagct	tatcgatgat	780
	aagcggtcaa	acatgagaat	togoggoogo	ataatacgas	tcactatagg	gatcgacgcc	840
15	tactccccgc	gcatgaagcg	gaggagctgg	actccgcatg	cccagagacg	cccccaacc	900
	cccaaagtgc	ctgacctcag	cctctaccag	ctctggcttg	ggcttgggcg	gggtcaaggc	960
	taccacgttc	tcttaacagg	tggctgggct	gtctcttggc	cgcgcgtcat	gtgacagctg	1020
	cctagttctg	cagtgaggtc	accgtggaat	gtctgccttc	gttgccatgg	caacgggatg	1080
	acgttacaat	ctgggtgtgg	agcttttcct	gtccgtgtca	ggaaatccaa	ataccctaaa	1140
20	ataccctaga	agaggaagta	gctgagccaa	ggctttcctg	gcttctccag	ataaagtttg	1200
	acttagatgg	aaaaaaacaa	aatgataaag	acccgagcca	tctgaaaatt	cctcctaatt	1260
	gcaccactag	gaaatgtgta	tattattgag	ctcgtatgtg	ttcttatttt	aaaaagaaaa	1320
	ctttagtcat	gttattaata	agaatttctc	agcagtggga	gagaaccaat	attaacacca	1380
	agataaaagt	tggcatgatc	cacattgcag	gaagatccac	gttgggtttt	catgaatgtg	1440
25	aagaccccat	ttattaaagt	cctaagctct	gtttttgcac	actaggaagc	gatggccggg	1500
	atggctgagg	ggctgtaagg	atctttcaat	gtcttacatg	tgtgtttcct	gtcctgcacc	1560
	taggacctgc	tgcctagcct	gcagcagagc	cagaggggtt	tcacatgatt	agtctcagac	1620
	acttgggggc	aggttgcatg	tactgcatcg	cttatttcca	tacggagcac	ctactatgtg	1680
	tcaaacacca	tatggtgttc	actcttcaga	acggtggtgg	tcatcatggt	gcatttgctg	1740
30	acggttggat	tggtggtaga	gagctgagat	atatggacgc	actcttcagc	attctgtcaa	1800
	cgtggctgtg	cattcttgct	cctgagcaag	tggctaaaca	gactcacagg	gtcagcctcc	1860
	agctcagtcg	ctgcatagtc	ttagggaacc	tctcccagtc	ctccctacct	caactatcca	1920
	agaagccagg	gggcttggcg	gtctcaggag	cctgcttgct	gggggacagg	ttgttgagtt	1980
	ttatctgcag	taggttgcct	aggcatagtg	tcaggactga	tggctgcctt	ggagaacaca	2040
35	tcctttgccc	tctatgcaaa	tctgaccttg	acatgggggc	gctgctcagc	tgggaggatc	2100
	aactgcatac	ctaaagccaa	gcctaaagct	tottogtoca	cctgaaactc	ctggaccaag	2160

	gggcttccgg	cacateetet	caggccagtg	agggagtctg	g tgtgagctg:	actttccaat	2220
						tecteccate	2290
						CCCaccccca	2340
					9999999999		2400
5					g Ctttgtaaga		2460
					gaccattcag		2520
					, acattgcaga		2580
					cctgacatgt		2640
	ttcttcaccc	agtcaccgaa	catttattca	gtacctaccc	cgtaacaggc	accgtagcag	2700
10					gccttggaat		2760
					ggccctttaa		2820
	ccccacccc	accccaagca	gttggcactg	ctatccacat	tttacagaga	ggaaaaacta	2880
	ggcacaggac	gatataagtg	gcttgcttaa	gcttgtctgc	atggtaaatg	gcagggctgg	2940
	attgagaccc	agacattcca	actctagggt	ctatttttct	tttttctcgt	tgttcgaatc	3000
15	tgggtcttac	tgggtaaact	caggctagcc	tcacactcat	atccttctcc	catggcttac	3060
	gagtgctagg	attccaggtg	tgtgctacca	tgtctgactc	cctgtagctt	gtctatacca	3120
	tcctcacaac	ataggaattg	tgatagcagc	acacacaccg	gaaggagctg	gggaaatccc	3180
					aaggtgggga		3240
	agggaacagc	atgggcgtgg	gaccacaagt	ctatttgggg	aagctgccgg	taaccgtata	3300
20	tggctggggt	gaggggagag	gtcatgagat	gaggcaggaa	gagccacagc	aggcagcggg	3360
					cttcctcctt		3420
					ctcaggacat		3480
					gcaggtcctt		3540
					acttgggcat		3600
25					ccacagattt		3660
	gcgctggccc	ataaaaatgg	taaggaacgt	acattccggc	acccatggag	cgtaagccct	3720
	ctgggacctg	cttcctccaa	agaggccccc	acttgaaaaa	ggttccagaa	agatcccaaa	3780
	atatgccacc	aactagggat	taagtgtcct	acatgtgagc	cgatgggggc	cactgcatat	3840
	agtetgtgee	atagacatga	caatggataa	taatatttca	gacagagagc	aggagttagg	3900
30	tagctgtgct	cctttccctt	taattgagtg	tgcccatttt	tttattcatg	tatgtgtata	3960
	catgtgtgtg	cacacatgcc	ataggttgat	actgaacacc	gtcttcaatc	gttccccacc	4020
	ccaccttatt	ttttgaggca	gggtctcttc	cctgatcctg	gggctcattg	gtttatctag	4080
	gctgctggcc	agtgagctct	ggagttctgc	ttttctctac	ctccctagcc	ctgggactgc	4140
					atctgaactt		4200
35					ttgagcaagt		4260
					ctaggcattc		4320

	acctogocaç	aggaatgagt	ggccacgact	ggctcaggg	t cagcageeta	a gagatactgg	4380
	gttaagtctt	cctgccgctc	geteeetge	gccgcagac	a gaaagtagga	Ctgaatgaga	4440
	gctggctagt	ggtcagacag	gacagaaggo	tgagagggt	c acagggcaga	tg:cagcaga	4500
	gcagacaggt	tatacatatg	tgggggaggg	gtggcccaci	t gcaggtgtaa	ttggccttct	4560
5	ttgtgctcca	tagaggcttc	ctgggtacac	agcagette:	c ctgtcctggt	gattoccaaa	4620
	gagaactccc	taccactgga	cttacagaag	ttctattgad	tggtgtaacg	gttcaacagc	4680
	tttggctctt	ggtggacggt	gcatactgct	gtatcagcto	aagagctcat	tcacgaatga	4740
	acacacacac	acacacacac	acacacacac	acacaagcta	attttgatat	gccttaacta	4800
	gctcagtgac	tgggcatttc	tgaacatccc	tgaagttago	acacatttcc	ctctggtgtt	4860
10	cctggcttaa	caccttctaa	atctatattt	tatctttgct	gccctgttac	cttctgagaa	4920
	gcccctaggg	CCACTTCCCT	tcgcacctac	attgctggat	ggtttctctc	ctgcagctct	4980
	taaatctgat	ccctctgcct	ctgagccatg	ggaacagccc	aataactgag	ttagacataa	5040
	aaacgtctct	agccaaaact	tcagctaaat	ttagacaata	aatcttactg	gttgtggaat	5100
	ccttaagatt	cttcatgacc	tccttcacat	ggcacgagta	tgaagcttta	ttacaattgt	5160
15	ttattgatca	aactaactca	taaaaagcca	gttgtctttc	acctgctcaa	ggaaggaaca	5220
	aaattcatcc	ttaactgatc	tgtgcacctt	gcacaatcca	tacgaatatc	ttaagagtac	5280
	taagattttg	gttgtgagag	tcacatgtta	cagaatgtac	agctttgaca	aggtgcatcc	5340
	ttgggatgcc	gaagtgacct	gctgttccag	ccccctacct	tctgaggctg	ttttggaagc	5400
20	aatgctctgg	aagcaacttt	aggaggtagg	atgctggaac	agcgggtcac	ttcagcatcc	5460
20	cgatgacgaa	tcccgtcaaa	gctgtacatt	ctgtaacaga	ctgggaaagc	tgcagacttt	5520
	aaggccaggg	ccctatggtc	cctcttaatc	cctgtcacac	ccaacccgag	cccttctcct	5580
	ccagccgttc	tgtgcttctc	actctggata	gatggagaac	acggccttgc	tagttaaagg	5640
	agtgaggett	caccettete	acatggcagt	ggttggtcat	cctcattcag	ggaactctgg	5700
25	ggcattctgc	ctttacttcc	tctttttgga	ctacagggaa	tatatgctga	cttgttttga	5760
25	ccttgtgtat	ggggagactg	gatctttggt	ctggaatgtt	tcctgctagt	ttttccccat	5820
	cctttggcaa	accctatcta	tatcttacca	ctaggcatag	tggccctcgt	tctggagcct	5880
	gccttcaggc	tggttctcgg	ggaccatgtc	cctggtttct	ccccagcata	tggtgttcac	5940
	agtgttcact	gcgggtggtt	gctgaacaaa	gcggggattg	catcccagag	ctccggtgcc	6000
20	ttgtgggtac	actgctaaga	taaaatggat	actggcctct	ctctgaccac	ttgcagagct	6060
30	ctggtgcctt	gtgggtacac	tgctaagata	aaatggatac	tggcctctct	ctatccactt	6120
	gcaggactct	agggaacagg	aatccattac	tgagaaaacc	aggggctagg	agcagggagg	6180
	tagctgggca	gctgaagtgc	ttggcgacta	accaatgaat	accagagttt	ggatctctag	6240
	aatactctta	aaatctgggt	gggcagagtg	gcctgcctgt	aatcccagaa	ctcgggaggc	6300
25	ggagacaggg	aatcatcaga	gcaaactggc	taaccagaat	agcaaaacac	tgagctctgg	6360
35	gctctgtgag	agatectgee	ttaacatata	agagagagaa	taaaacattg a	aagaagacag	6420
	tagatgccaa	ttttaagccc	ccacatgcac	atggacaagt	gtgcgtttga a	acacatat	6480

	gcactcatgt	gaaccaggca	FOCACACEC	~~~=====			
						: tgaaagagag	6540
						acacccatgo	6600
						agagggagcc	6660
5						actggagcat	6720
5						cagagggtcc	6780
						gccagagaga	6840
						gcaagtagag	6900
			ggggtgcaga				6960
10						ctcagcagag	7020
10			tgctggaaat				7080
			gtggatctgt				7140
			actgggtgat				7200
	tggcctgggg	ctttgtttct	gtctctgttt	tgtttcgttt	tttgagacag	actcttgcta	7260
	tgtatccgtg	tcaatcttgg	aatctcactg	catageceag	gctgcggaga	gaggggaggg	7320
15	caataggcct	tgtaagcaag	ccacacttca	gagactagac	tccaccctgc	gaatgatgac	7380
	aggtcagagc	tgagttccgg	aagattttt	ttccagctgc	caggtggagt	gtggagtggc	7440
			ggcgagctcc				7500
	caagccagtg	agttaagcat	tctgtgtggg	gagcaggtgg	atgaagagag	aggctgggct	7560
	ttegeetetg	gggggggt	gaggggtggg	gatgaggtga	gaggagggca	gctccctgca	7620
20	gtgtgatgag	atttttcctg	acagtgacct	ttggcctctc	cctccccac	ttcccttctt	7680
			tttccttgtc				7740
			agccgtgtgt				7800
	gtgtgtgtgt	ttgtgtgtat	gtgtgtgtgt	gtgtttgtgt	gtatgtgtgt	cagtgggaat	7860
			gtgggcagga				7920
25			gagggatacc				7980
			tgtgtgtgtt				8040
	taaaaaatac	ttatccattt	atttatttt	atgtgcacgt	gtgtgtgcct	gcatgagttc	8100
	atgtgtgcca	cgtgtgtgcg	ggaacccttg	gaggccacaa	gggggcatct	gatcccctgg	8160
			gtgagtcccc				8220
30			gcagttatct				8280
			cagagacatg				8340
			gcaagcacct				8400
			ttcttcatgt				8460
			togotggaga				8520
35			cccttcctgg				8580
			ctctcgctct				8640
							2040

	gtgtatgcac	atgtgccaca	tgtgtacaga	tactatggag	g gccagaagag	gccatggccg	8700
					g gtgtgggtgd		8760
					totoagtaco		8820
					a atatottgot		8880
5					g cctgagctgg		8940
					, atgacaggcg		9000
					tcatacagag		9060
					caagaaacaa		9120
					ttcaattccc		9180
10					ttctggtgtg		9240
	gctacagtgt	actcacataa	aataaataaa	tctttaaaac	acacacacac	acacaattac	9300
	caccccagaa	agcccactcc	atgttccctc	ccacgtctct	gcctacagta	ctcccaggtt	9360
	accactgttc	aggcttctaa	caacctggtt	tacttgggcc	tcttttctgc	tctgtggagc	9420
	cacacatttg	tgtgcctcat	acacgttctt	tctagtaagt	tgcatattac	tctgcgtttt	9480
15	tacatgtatt	tatttattgt	agttgtgtgt	gcgtgtgggc	ccatgcatgg	cacagtgtgt	9540
	ggggatgtca	gagtattgtg	aacaggggac	agttcttttc	ttcaatcatg	tgggttccag	9600
					tacccactga		9660
					gcccaaactg		9720
					ttggaattac		9780
20					ggaagcgcac		9840
					gaacagcttc		9900
					acagaaagtt		9960
					tggctcctct		10020
					aaaaagactt		10080
25					ccagaggtcc		10140
					tcaacgtgag		10200
	cagttttccg	cggtggagaa	cctcttgaca	ccctgctgtc	cctggtcatt	ctgggtgggt	10260
					acagtgaagt		10320
• 0					tgtccattgc		10380
30					gcggcagaag		10440
					ccccaacgat		10500
					caaggtgaaa		10560
					ggcagccatg		10620
					atctgtccca		10680
35					cctaagaagt		10740
	agccatcctt	tcctgtaatt	tatgtctctc	cctgaggtga	ggttcaggtt	tatgtccctg	10800

	tctgtggcat	agatacatc	t cagtgacco	a gggtgggag	gg gctatcagg	g tgcatggccc	10860
	gggacacggg	cactettea	t gacccctcc	c ccacctggg	t tottootgt	g tggtccagaa	10920
	ccacgagcct	ggtaaagga	a ctatgcaaa	c acaggeeet	g acctecesa	E Giotaltech	10980
	ggtcctcaca	gcccgacac	g coptgotga	g gcagacgaa	t gacattaag	t totgaagcag	11040
5	agtggagata	gattagtga:	tagattico	a aaaagaagg	a aaaaaagg	C tgcattttaa	11100
	aattatttcc	ttagaattaa	a agatactac	a taggggccc	t tgggtaagc	a aatccattt	11160
	Ecccagaggc	tatcttgatt	ctttggaat	g tttaaagtg	t gccttgccad	agagetaca	11220
	atctatatct	gctgcttcag	agcetteect	gaggatggc	t ctgttccttt	gottatraga	11280
	agagcgatgc	cttgggcagg	gtttacccat	tttcagaat	a cagggtgtaa	agtocadoor	11340
10	attacaaaca	aacaaacaaa	caaacaaaca	a aaggacctc	atttggagaa	i ttqcaaqqar	11400
	tttatcctga	attatagtgt	tggtgagtto	aagtcatca	gccaagtgct	tgccatccta	11460
	gttgctattc	taagaataat	taggaggagg	aacctagcca	attgcagete	atgtccgraa	11520
	gtgtgtgcac	gggtgcatat	gttggaaggg	gtgcctgtcd	ccttggggac	agaaggaaaa	11580
	tgaaaggccc	ctctgctcac	cctggccatt	tacgggaggc	totgotgatt	CCacagraro	11640
15	tgtgcaggat	cctgaaactg	actcgctgga	cagaaacgag	acttggcggc	accatgagaa	11700
	rggagagaga	gagagcaaag	aaagaaacag	cctttaaaag	aactttctaa	gggtggtr++	11760
	Lgaacctcgc	tggaccttgt	atgtgtgcac	atttgccaga	gattgaacat	aatcctctta	11820
	ggacttcacg	ttctcattat	ttgtatgtct	ccggggtcac	gcagagccgt	Cagodaccac	11880
20	cccagcaccc	ggcacatagg	cgtctcataa	aagcccattt	tatgagaacc	agaggrafff	11940
20	gagtaccccg	tgtatagaga	gagttgttgt	cgtggggcac	ccggatccca	gcagcctggt	12000
	rgeetgeetg	taggatgtct	tacaggagtt	tgcagagaaa	ccttccttag	agggaaagaa	12060
	atatcaggga 1	tttttgttga	atatttcaaa	ttcagcttta	agtgtaagac	tcagcagtgt	12120
	tcatggttaa	ggtaaggaac	atgccttttc	cagagetget	gcaagaggca	ggagaagcag	12180
25	acctgtctta g	ggatgtcact	cccagggtaa	agacctctga	tcacagcagg	agcagagctg	12240
	tgcagcctgg a	arggreatig	tcccctattc	tgtgtgacca	cagcaaccct	ggtcacatag	12300
	ggctggtcat d		tttttttt	ttttttttg	gcccagaatg	aagtgaccat	12360
	agccaagttg t	gracercag	tctttagttt	ccaagcggct	ctcttgctca	atacaatgtg	12420
	catttcaaaa t	aacactgta	gagttgacag	aactggttca	tgtgttatga	gagaggaaaa	12480
30	gagaggaaag a	acaaaacaa	aacaaacac	cacaaaccaa	aaacatctgg	gctagccagg	12540
	catgattgca a	agatacag	gcccagttca	tgagaggcag	agacaggaag	accgccgaaa	12600
	ggtcaaggat a	gcatggtct	acgtatcgag	actccagcca	gggctacggt	cccaagatcc	12660
	taggttttgg a	ractates	ttggtttttg	agacagggtt	tetetgtgta	gccctggctg	12720
	tcctggaact c	getetgtag .	accaggctgg	cctcaaactt	agagatetge (ctgactctgc	12780
35	ctttgagggc t	yyyacgaat (gccaccactg	cccaactaag	attccattaa a	aaaaaaaa	12840
	agttcaagat a	ctottott	rgccagctcg	ttaaagctaa	gtagaagcag t	ctcaggcct	12900
	gctgcttgag g	ergredeng (guntggaddt (gaaatctgcc	cccaacagtg t	ccaagtgca	12960

	catgactttc	agccatctco	agagaagga:	a grgaaaar	7	a gtcgattggg	
	acacagtete	totttgtota	ggtaacaca	- grydddici	# Regare	a gicgatiggg c telecacter	13020
	gagggtgggt	ttccctccc	Ctacctctt	- sgrgaede	S agrantes	g acagecacag	13080
	gacagtcact	agcacctact	ggaaacctc	ttaraas	C accedatag	g acagccacag g agcctttggg	13140
5	agattcctgg	- Ctttccatta	gggctgaaa	T Facaaccat	t atgaagaaa	g agcetttggg e tttgeetegt	13200
	gtttataaaa	Ctaqctacta	LECTICACCE	· aaaataacggt	c cttggttgg	c tttgcctcgt	13260
	cgtqqctqcc	CGtgagtag	aggt gaget	. aaaacaccg	a tgttgtgga	a aagccaaccc	13320
	aaqtqqtqqa	ataggaatta	3991999911	gggaatttt	g gatagtgtt	c tatccatgga	13380
	CCACTTTCTA	tgactrataa	3635663666		c aacctcttc	c tcagacccag	13440
10	totcaarorr	Clasagecta	Conserva	aaaaattac	a aacataaaa	tggtttctct	13500
	attororroa	gaggagagag	tangeettt	ccaggggtag	g gtctgtttc:	ttgctgttct	13560
	getetterag	gctccagac	taacacetac	caaatgagg	g aactcttggo	ccatactaag	13620
	accorded	CCCBBCEC	tettaagtta	ttttaagaat	teteacttg] cctttagcac	13680
	gggtgccttt	cccaagiggg	rgrggataat	gccatggcca	a gcagggggca	ctgttgaggc	13740
15	gaagagtga	tettetaag	ttgcttatag	tatttaagat	gctaaatgtt	ttaatcaaga	13800
	taattottt	CCCCacaca	cgaggataag	agattttctc	acaggaaatt	gtctttttca	13860
	tatattaan	acaggeeteg	tectgategt	agcatagaga	gaatagetge	atatttaact	13920
	gaagagaga	tttcctctgc	cagcgttagg	ttaactccgt	aaaaagtgat	tcagtggacc	13980
	gaagaggctc	agagggcagg	ggatggtggg	gtgaggcaga	gcactgtcac	ctgccaggca	14040
20	tgggaggtcc	tgccatccgg	gaggaaaagg	aaagtttago	ctctagtcta	ccaccagtgt	14100
20	taacgcactc	taaagttgta	accaaaataa	atgtcttaca	ttacaaagac	gtctgttttg	14160
	tgtttddtt	tgtgtgtttg	ggctttttat	gtgtgcttta	taactgctgt	ggtggtgctg	14220
	ttgttagttt	tgaggtagga	tctcaggctg	gccttgaact	tctgatcgcc	tgcccctgcc	14280
	cctgcccctg	cccctgtccc	tgcctccaag	tgctaggact	aaaagcacat	gccaccacac	14340
25	cagtacagca	tttttctaac	atttaaaaat	aatcacctag	gggctggaga	gagggttcca	14400
25	gctaagagtg	cacactgctc	ttgggtagga	cctgagttta	gttcccagaa	cctatactgg	14460
	gtggctccag	gtccagagga	tccaggacct	ctggcctcca	tgggcatctg	Ctcttagcac	14520
	atacccacat	acagatacac	acataaaaat	aaaatgaagc	Ctttaaaaac	Ctcctaaaac	14580
	ctagcccttg	gaggtacgac	tctggaaagc	tggcatactg	tgtaagtcca	tctcatagta	14640
••	ttctggctaa	cgtaagactt	acagagacag	aaaagaactc	agggtgtgct	gagaattaaa	14700
30	atggaggaag	agggatgagt	agggggagca	cggggaactt	gggcagtgaa	aattctttgc	14760
	aggacactag	aggaggataa	ataccagtca	ttgcacccac	tactggacaa	Ctccagggaa	14820
	ttatgctggg	tgaaaagaga	aggccccagg	tattggctgc	attggctgca	tttgcgtaac	14880
	attttttaa	attgaaaaga	aaaagatgta	aatcaaggtt	agatgagtgg	ttqctqtqaq	14940
	ctgagagctg	gggtgagtga	gacatgtgga	caactccatc	aaaaagcgac	agaaagaacg	15000
35	ggctgtggtg a	acagctacct	ctaatctcca	cctccgggag	gtgatcaagg	ttagecetea	15060
	gctagcctgt	ggtgcatgag a	accctgtttc	aaaaacttta	ataaaqaaat	aatgaaaaaa	15120
					3		13120

	gacatcaggg cagat	CCtta qaaccaaa	ia caassaas	20.		
	tagaagcgga tgcat	gagca cotoccoca	ag cggtcaggt	e agectegte	g taaggtegtg	15180
	atggatgtga gtgtg	Ecado attacada	s tacacara	g agageceta	g gtaagtaagg	15240
	atotttqqtq aqctq	taasa qaqaaataa	s tageacyte	t ggetgtggt -	g ctggactggc	15300
5	atctttggtg agctg	TTACA ATTACCES	g cagggagae	c ataaaatcc	c toogaattat	15360
	ttcaagaact gtcta	atara ataatata	a datattaaa	a aaaaagaag	a attaaaaaac	15420
	aaaaaaccta tccagg	orten aangtatet	a colatages	a cgggcactt	g gaaagctgga	15480
	gcaagaggat ggcgag	Bacco attatoto	g gggctgtac	a gcaagaccg	t cgtccccaaa	15540
	gagagcatgg ggtgg	ages gagarages	c acaagagtg	t ttatagtgag	g cggcctcgct	15600
10	gagagcatgg ggtggg	gaaca gaggigggg	g acagaaata	t ctaaactgca	gtcaataggg	15660
	atccactgag accets	agge tigactgca	g cttaacctt	g ggaaatgata	agggttttgt	15720
	gttgagtaaa agcato	gatt actgactta	a cctcaaatg	a agaaaaagaa	a aaaaagaaaa	15780
	caacaaaagc caaacc	aagg ggctggtga	g atggeteag:	gggtaagago	accogactgo	15840
	tottccgaag gtccag	agtt caaatccca	g caaccacate	g gtggctcaca	accatctgta	15900
15	acgagatatg atgccc	:Idtt ctggtgtgtd:	tgaagacag	tacagtgtac	ttacatataa	15960
	taaataaatc ttaaaa	aaaa aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa	a aaaagccaaa	a ccgagcaaac	caggccccca	16020
	aacagaaggc aggcac	gacg gcaggcacca	a cgagccatco	tgtgaaaagg	cagggctacc	16080
	catgggccga ggaggg	tcca gagagatago	g ctggtaagct	cagtttctct	gtataccctt	16140
	tttcttgttg acacta	cttc aattacagat	aaaataacaa	ataaacaaaa	tctagagcct	16200
20	ggccactete tgeteg	cttg atttttcctg	, ttacgtccag	caggtggcgg	aagtgttcca	16260
20	aggacagate gcatca	ttaa ggtggccago	ataatctccc	atcagcaggt	ggtgctgtga	16320
	gaaccattat ggtgct	caca gaatcccggg	cccaggaget	gccctctccc	aagtctggag	16380
	caataggaaa gctttc	tggc ccagacaggg	ttaacagtcc	acattccaga	gcaggggaaa	16440
	aggagactgg aggtca	caga caaaagggco	agcttctaac	aacttcacag	ctctggtagg	16500
25	agagatagat cacccc	caac aatggccaca	gctggttttg	tctgccccga	aggaaactga	16560
23	cttaggaagc aggtato	caga gtccccttcc	tgaggggact	tctgtctgcc	ttgtaaagct	16620
	gtcagagcag ctgcatt	tgat gtgtgggtga	cagaagatga	aaaggaggac	ccaggcagat	16680
	cgccacagat ggaccgg	JCCa Cttacaagtc	gaggcaggtg	gcagagcctt	gcagaagctc	16740
	tgcaggtgga cgacact	gat tcattaccca	gttagcatac	cacagcgggc	taggcggacc	16800
20	acageeteet teecagt	ctt cctccagggc	tggggagtcc	tccaaccttc	tqtctcaqtq	16860
30	cagetteege cageeee	tcc tccttttgca	cctcaggtgt	gaaccctccc	tcctctcctt	16920
	ctccctgtgg catggco	ctc ctgctactgc	aggctgagca	ttggatttct	ttgtgcttag	16980
	atagacctga gatggct	ttc tgatttatat	atatatatcc	atcccttgga	tottacator	17040
	aggacccaga gctgttt	gtg ataccataag	aggctgggga	gatgatatgg	taagagtgcr	17100
2.5	tgctgtacaa gcatgaa	gac atgagttcga	atccccagca	accatgtgga	aaaataacct	17160
35	tctaacctca gagttga	ggg gaaaggcagg	tggattctgg	gggcttactg	gccagctagc	17220
	cagcctaacc taaatgt	ctc agtcagagat	cctgtctcag	ggaataactt	gggagaatga	17280

	ctgagaaaga cacstootoa ggtotoocat gcascoacas agasacasgg gggggggta	17340
	atgtaataag ctaagaaata atgagggaaa tgattttttg ctaagaaatg aaattctgtg	17400
	tiggeegeaa gaageetgge cagggaagga actgeettig geacaceage etataagtea	17460
	ccatgagite ceiggetaag aateacaigi aatggageee aggiceetet igeeiggige	17520
5	tigectetee caetggitti gaagagaat teaagagaga teteetiggi caqaatigia	17580
	ggrgcrgage aargrggage rggggreaar gggarreert raaaggeare erreecaggg	17640
	cigggicata circaatagi agggigciig cacagcaagc gigagaccci aggilagagi	17700
	ccccagaatc tgcccccaac cccccaaaaa ggcatccttc tgcctctggg tgggtgggg	17760
•	gagcaaacac ctitaactaa gaccattagc tggcaggggt aacaaatgac cttggctaga	17820
10	ggaattiggt caagetggat teegeettet gtagaageee caettgitte ettigttaag	17880
	ctggcccaca gtttgttttg agaatgcctg aggggcccag ggagccagac aattaaaagc	17940
	caageteatt tigatateig aaaaceaeag egigaeigee eigeeegigg gaggiacigg	18000
	gagagetgge tgtgteeetg eeteaceaac geeeeeeee eeaacacae etectegge	18060
1.5	caccigggag gigccagcag caaitiggaa giitacigag ciigagaagi ciigggaggg	18120
15	ctgacgetaa geacaceeet tetecaceee ceeecaceee acceeegtga ggaggaggg	18180
	gaggaaacat gggaccagec ctgeteeage cegteettat tggetggeat gaggeagagg	18240
	gggetttaaa aaggeaaceg tatetagget ggacaetgga geetgtgeta eegagtgeee	18300
	tectecacet ggeageatge ageceteact ageceegtge eteatetgee tacttgtgea	18360
20	cgctgccttc tgtgctgtgg agggccaggg gtggcaagcc ttcaggaatg atgccacaga	18420
20	ggtcatccca gggcttggag agtaccccga gcctcctcct gagaacaacc agaccatgaa	18480
	ccgggcggag aatggaggca gacctcccca ccatccctat gacgccaaag gtacgggatg	18540
	aagaagcaca ttagtggggg ggggggtcct gggaggtgac tggggtggtt ttagcatctt	18600
	cttcagaggt ttgtgtgggt ggctagcctc tgctacatca gggcagggac acatttgcct	18660
25	ggaagaatac tagcacagca ttagaacctg gagggcagca ttggggggct ggtagagagc	18720
••	acccaaggca gggtggaggc tgaggtcagc cgaagctggc attaacacgg gcatgggctt	18780
	gtatgatggt ccagagaatc tcctcctaag gatgaggaca caggtcagat ctagctgctg	18840
	accagtgggg aagtgatatg gtgaggctgg atgccagatg ccatccatgg ctgtactata	18900
	tcccacatga ccaccacatg aggtaaagaa ggccccagct tgaagatgga gaaaccgaga	18960
30	ggctcctgag ataaagtcac ctgggagtaa gaagagctga gactggaagc tggtttgatc	19020
	cagatgcaag gcaaccctag attgggtttg ggtgggaacc tgaagccagg aggaatccct	9080
	ttagttcccc cttgcccagg gtctgctcaa tgagcccaga gggttagcat taaaagaaca	9140
	gggtttgtag gtggcatgtg acatgagggg cagctgagtg aaatgtcccc tgtatgagca 1	9200
	caggiggeac cactigeeet gagetigeac eetgaceea gettigeete atteetgagg 1	9260
35	acagcagaaa ctgtggaggc agagccagca cagagagatg cctggggtgg gggtgggggt 1	9320
	atcacgcacg gaactagcag caatgaatgg ggtggggtgg	9380
	agaaatgacc ttgctggtca ccatttgtgt gggaggagag ctcattttcc agcttgccac 1	9440

	cacatgcigt coetectgic testagecag taagggatgt ggaggaaagg gccaccccaa	
	aggagcatge aatgeagtea egittitgea gaggaagtge tigacetaag ggeactatte	19500
	ttggaaagee ccaaaactag teetteeetg ggeaaacagg ceteeceac ataccacete	19560
	tgcaggggtg agtaaattaa gccagccaca gaagggtggc aaggcctaca cctccccct	19620
5	grigigeded ecoececec graaaggrad atecrageer ergedeeter ggerriggta	19680
	ctgggatttt ttttttcctt ttatgtcata ttgatcctga caccatggaa cttttggagg	19740
	tagacaggac ccacacatgg attagttaaa agcctcccat ccatctaagc tcatggtagg	19800
	agatagagca tgtccaagag aggagggcag gcatcagacc tagaagatat ggctgggcat	19860
	ccaacccaat ctccttcccc ggagaacaga ctctaagtca gatccagcca cccttgagta	19920
10	accageteaa ggtacacaga acaagagagt etggtataca geaggtgeta aacaaatget	19980
	tgtggtagca aaagctatag gttttgggtc agaactccga cccaagtcgc gagtgaagag	20040
	cgaaaggeee tetactegee acegeeeege ceecacetgg ggteetataa cagateaett	20100
	tcaccettge gggagecaga gagecetgge atcetaggta gecececeg eccecece	20160
	gcaagcagcc cagccctgcc tttggggcaa gttcttttct cagcctggac ctgtgataat	20220
15	gagggggttg gacgcgccgc ctttggtcgc tttcaagtct aatgaattct tatccctacc	20280
	acctgccctt ctaccccgct cctccacage agetgtcctg atttattace ttcaattaac	20340
	ctccactcct ttctccatct cctgggatac cgcccctgtc ccagtggctg gtaaaggagc	20400
	ttaggaagga ccagagccag gtgtggctag aggctaccag gcagggctgg ggatgaggag	20460
	ctaaactgga agagtgtttg gttagtaggc acaaagcett gggtgggate ectagtaceg	20520
20	gagaagtgga gatgggcgct gagaagttca agaccatcca tccttaacta cacagccagt	20580
	ttgaggccag cctgggctac ataaaaaccc aarctcaaaa cor	20640
	gccacgtagt gcccgatgta aragtggatg aagtggtta	20700
	acagatgtgg ggaaaaqcaa ctttaagtac cctgcccasa gabaasa	20760
	acagagetee agtgttteat ceetgggtte caaggagaga gaaaaaa	20820
25	atctcactgc tccccqqtqc ctccttccta taatccatag access	20880
	ggtttggaaa aagagagaag ggtggaagga ggagaggagt gtggaaga	20940
	teaegeatee eteteteege agatgtetee gagtacaget gagggeren	21000
	egetteetga cagaeggeee atgeegeage gecaageegg teaceants	21060
	ggccagigcg gccccqcqcq gcigciaccc aacqccatca manaca	21120
30	ccgaacggac cggatttccg ctgcatcccg gatcgctaco	21180
	ctgtgccccg ggggcgcqcc gccgcgctca cgcaaggtgc gtctaaaa	21240
	tgcaagcgcc tcacccgctt ccacaaccag tcggagctca aggacttcgg gccggagacc	21300
	gcgcggccgc agaagggtcg caagccgcgg cccggcgcg garages	21360
	geggagetgg agaacgeeta etagagegag eeegggggaa anaaa	21420
35	attogette agegraaage etgeageera ggeraggger anna an	21480
	tggagttece ageceagtag agacegeagg teettetggg agetter	21540
	-	21600

	000F0000FF	555555555					
						ctageceegg	21650
						g tgtttccacc	21720
						acagtaagat	21780
						gaccotgaco	21840
5						tctttgtaaa	21900
						gactggtgag	21960
						cctagaagaa	22020
						ctccttcctc	22080
	tcaaatctgc	cttcaaatcc	atatctggga	tagggaaggc	cagggtccga	gagatggtgg	22140
10	aagggccaga	aatcacactc	ctggcccccc	gaagagcagt	gtcccgccc	caactgcctt	22200
	gtcatattgt	aaagggattt	tctacacaac	agtttaaggt	cgttggagga	aactgggctt	22260
	gccagtcacc	tcccatcctt	gtcccttgcc	aggacaccac	ctcctgcctg	ccacccacgg	22320
	acacatttct	gtctagaaac	agagcgtcgt	cgtgctgtcc	tctgagacag	catatottac	22380
	attaaaaaga	ataatacggg	aaaaaaaaac	ggagggcgca	agtgttatac	atatgctgag	22440
15	aagctgtcag	gcgccacagc	accacccaca	atctttttgt	aaatcatttc	cagacacctc	22500
	ttactttctg	tgtagatttt	aattgttaaa	aggggaggag	agagagcgtt	tgtaacagaa	22560
	gcacatggag	ggggggtag	gggggttggg	gctggtgagt	ttggcgaact	ttccatgtga	22620
	gactcatcca	caaagactga	aagccgcgtt	tttttttta	agagttcagt	gacatattta	22680
	ttttctcatt	taagttattt	atgccaacat	ttttttcttg	tagagaaagg	cagtgttaat	22740
20	atcgctttgt	gaagcacaag	tgtgtgtggt	tttttgttt	ttgttttttc	cccgaccaga	22800
					gtggtcttgt		22860
	acacacaatg	tctcgccact	gtcatctcac	tcccttccct	tggtcacaag	acccaaacct	22920
	tgacaacacc	tccgactgct	ctctggtagc	ccttgtggca	atacgtgttt	cctttgaaaa	22980
					cccagctggg		23040
25	accctcaccc	cagcctccct	ttagctgacc	actctccaca	ctgtcttcca	aaagtgcacg	23100
	tttcaccgag	ccagttccct	ggtccaggtc	atcccattgc	tcctccttgc	tccagaccct	23160
	tctcccacaa	agatgttcat	ctcccactcc	atcaagcccc	agtggccctg	cggctatccc	23220
					gaattccttc		23280
					ctgtcccagc		23340
30	tcctctctct	tgtaaagccc	caccccacta	tttgattccc	aattctagat	cttcccttgt	23400
	tcattccttc						23460
	caaagccaag						23520
	ttttacacct						23580
	caacatcagg						23640
35	aggggtgtga						23700
	tcaacctctc						23760
					-	3	

	Caacacttra aarccagton notonen	
	caacacttta aatocagtca agigcatoti tgcgtgaggg gaactctato cotaatataa 23	820
	gcttccatct tgatttgtgt atgtgcacac tgggggttga acctgggcct ttgtacctgc 23	880
	cgggcaaget etetactget etaaacecag ceetcactgg etttetgttt caacteecaa 23	940
5	tgaattcccc taaatgaatt atcaatatca tgtctttgaa aaataccatt gagtgctgct 24	, 000
,		060
	aatgtettag agcaggagge catggagace ttggeeagee ceacaaggea gtgtggtgea 24:	20
	gagggtgagg atggaggcag gcttgcaatt gaagctgaga cagggtactc aggattaaaa 241	.80
	agetteecee aaaacaatte caagateagt teetggtaet tgeacetgtt cagetatgea 242	4 0
10	gageceagtg ggeataggtg aagaeaeegg ttgtaetgte atgtaetaae tgtgetteag 243	00
10	agccggcaga gacaaataat gttatggtga ccccagggga cagtgattcc agaaggaaca 243	60
	cagaagagag tgctgctaga ggctgcctga aggagaaggg gtcccagact ctctaagcaa 244	20
	agactecaet cacataaaga cacaggetga geagagetgg cegtggatge agggageeca 244	80
	tocaccatoc titagoatgo cottgiatio coatcacaig coagggatga ggggcaicag 245	40
	agagtecaag tgatgeecaa acceaaacae acetaggaet tgetttetgg gacagacaga 246	00
15	tgcaggagag actaggttgg gctgtgatcc cattaccaca aagagggaaa aaacaaaaaa 246	60
	caaacaaaca aacaaaaaaa aacaaaacaa aacaaaaaa	20
	ggtcaggtta gagtttattt atggaaagtt atattctacc tccatggggt ctacaaggct 247	30
	ggcgcccatc agaaagaaca aacaacaggc tgatctggga ggggtggtac tctatggcag 248	1 0
	ggagcacgig igcitggggi acagccagac acggggcitg tattaatcac agggcitgia 2490	00
20	ttaatagget gagagteaag cagacagaga gacagaagga aacacacaca cacacaca	50
	cacacacaca cacacaca catgeacaca ecaeteaett eteaetegaa gageecetae 2502	
	ttacattota agaacaaaco attootooto ataaaggaga caaagttgoa gaaacccaaa 2508	30
	agagecacag ggteeccaet etetttgaaa tgaettggae ttgttgeagg gaagaeagag 2514	
	gggtctgcag aggcttcctg ggtgacccag agccacagac actgaaatct ggtgctqaqa 2520	10
25	cctgtataaa ccctcttcca caggttccct gaaaggagcc cacattcccc aaccctgtct 2526	
	conducted control of the control of	
	ccccatctga gggcacatga ggtctcaggt cttgggaaag ttccacaagt attgaaagtg 2538	
	ttottgtttt gtttgtgatt taatttaggt gtatgagtgc ttttgcttga atatatgcct 2544	
	gtgtagcatt tacaagcctg gtgcctgagg agatcagaag atggcatcag ataccctgga 2550	
30	actggacttg cagacagtta tgagccactg tgtgggtgct aggaacagaa cctggatcct 2556	•
	ccggaagagc agacagccag cgctcttagc cactaagcca tcactgaggt tctttctgtg 2562	
	gctaaagaga caggagacaa aggagagttt cttttagtca ataggaccat gaatgttcct 2568	
	cgtaacgtga gactagggca gggtgatccc ccagtgacac cgatggccct gtgtagttat 2574	=
	tagcagetet agtettatte ettaataagt eecagtttgg ggeaggagat atgtattee 25800	
35	tgctttgaag tggctgaggt ccagttatct acttcgaagt actaga	
	ttggggaage teeetgeetg cetgraaate tgtgeattet talle	
	25920	,

	tttccctgag	g cagtcaggc	agtccaaag	ccitcaatt	t agettteat	a aggaacaccc	25980
	cttttgttgg	gtggaggtag	g cacttgcctt	gaatcccag	c attaagaag	g cagagacagt	26040
	cggatctctc	g tgagttcaca	gccagcctgg	, totacggag	t gagttccaa	g acagecagge	26100
	ctacacagag	aaaccctgto	togaaaaaa	caaaaacaa	a agaaataaa	g aaaaagaaaa	26160
5	caaaaacgaa	caaacagaaa	a acaagccag	agtgtttgt	c cccgtattt	t attaatcata	26220
	tttttgtccc	tttgccattt	tagactaaaa	gactcggga	a agcaggtot	c tototgitto	26280
	tcatccggac	acacccagaa	ccagatgtat	ggaagatgg	c taatgtgct	g cagttgcaca	26340
	tctggggctg	ggtggattgg	ttagatggca	tgggctggg	t gtggttacga	tgactgcagg	26400
	agcaaggagt	atgtggtgca	tagcaaacga	ggaagtttg	acagaacaac	actgtgtgta	26460
10	ctgatgtgca	ggtatgggca	catgcaagca	gaagccaag	g gacageetta	gggtagtgtt	26520
	tccacagacc	cctccccct	tttaacatgg	gcatctctca	ttggcctgga	gettgecaac	26580
	rgggctgggc	tggctagctt	gtaggtccca	gggatctgca	tatctctgcc	tecetagies	26640
	tgggattaca	gtcatatatg	agcacacctg	gcttttttat	gtgggttctg	ggctttgaac	26700
	ccagatctga	gtgcttgcaa	ggcaatcggt	tgaatgactg	cttcatctcc	ccagaccctq	26760
15	ggattctact	ttctattaaa	gtatttctat	taaatcaatg	agcccctgcc	cctqcactca	26820
	gcagttctta	ggcctgctga	gagtcaagtg	gggagtgaga	gcaagcctcg	agaccccatc	26880
	agcgaagcag	aggacaaaga	aatgaaaact	tgggattcga	ggctcgggat	atggagatac	26940
	agaaagggtc	agggaaggaa	atgaaccaga	tgaatagagg	caggaagggt	agggccctgc	27000
20	atacatggaa	cctggtgtac	atgttatctg	catggggttt	gcattgcaat	ggctcttcag	27060
20	caggitcacc	acactgggaa	acagaagcca	aaaagaagag	taggtggtgt	tggagtcaga	27120
	tactgtcagt	catgcctgaa	gaaatggaag	caattaacga	tgcgccgcaa	ttaggatatt	27180
	agctccctga	agaaaggcaa	gaagctgggc	tgtgggcact	gaagggagct	ttgaatgatg	27240
	tcacattctc	tgtatgccta	gcagggcagt	attggagact	gagacttgac	ttgtgtgtcc	27300
25	atatgattcc	tocttttcct	acagtcatct	ggggctcctg	agcttcgtcc	ttgtccaaga	27360
43	acctggagct	ggcagtgggc	agctgcagtg	atagatgtct	gcaagaaaga	tctgaaaaga	27420
	gggaggaaga	tgaaggaccc	agaggaccac	cgacctctgc	tgcctgacaa	agctgcagga	27480
	agazza	ctacagatgg	gagacagagg	cgagagatga	atggtcaggg	gaggagtcag	27540
	agaaaggaga	gggtgaggca	gagaccaaag	gagggaaaca	cttgtgctct	acagctactg	27600
30	actgagtacc	agctgcgtgg	cagacagcca .	atgccaaggc	tcggctgatc	atggcacctc	27660
50	gtgggactcc	tagcccagtg	ctggcagagg (ggagtgctga	atggtgcatg	gtttggatat	27720
	gatctgaatg	tggtccagcc	ctagtttcct 1	tccagttgct	gggataaagc	accctgacca	27780
	aagctacttt	tttgtttgtt	tgttttggtt i	ggttttgtt	tggtttttcg	aggcagggtt	27840
	tctctgtatc a	accctagctg	tcctggaact (cactctgtag	accaggctgg	cctcgaactc	27900
	agaaatcccc (ctgcctctgc	ctcctaagtg (tggaattaa	aggcctgcgc	caccactgcc	27960
35	ggcccaaagc t	cactttaaga (gagagagagg a	aatgtataag	tattataatt	ccaggttata	28020
	gttcattgct o	gtagaattgg a	agtcttcata t	tccaggtaa	tctcccacag a	acatgccaca	28080

	aaacaacctg tictacgaaa teletcatgg actocctice ccagtaatte taaactgtgt	28140
	Caaatetaca agaaatagtg acagtcacag tetetaaegt titigggeatg agtergaagt	28200
	ctcattgcta agtactggga agatgaaaac tttacctagt gtcagcattt ggagcagage	28260
	ctttgggatt tgagatggtc ttttgcagag ctcctaatgg ctacatggag agaggggg	28320
5	rgggagagad coatacacci titgoigest taigicacci gaccigoice tigggaage:	28380
	ctagcaagaa ggccttccct ggatcaccca ccaccttgca cctccagaac tcagagccaa	28440
	attaaacttt cttgttactg tcgtcaaagc acagtcggtc tgggttgtat cactgtcaar	28500
	gggaaacaga cttgcctgga tggataactt gtacattgca taatgtctag aaatgaaaag	28560
	teetatagag aaaaagaaaa ttagetggea cacagataga ggeeetggag gaggetgger	28620
10	tigiccicco cgaggaggig gcgagtaagg igiaaaigii caiggaigta aaigggcca	28680
	tatatgaggg tetggggtaa caagaaggee tgtgaatata aageaetgaa ggtatgreta	28740
	gtotggagaa ggtoactaca gagagttoto caactoagtg cocatacaca cacacacaca	28800
	Cacacacaca cacacacaca cacacacaca ccacaaagaa aaaaaggaag aaaaatcrga	28860
1.5	gagcaagtac agtacttaaa attgtgtgat tgtgtgtgtg actctgatgt cacatgorca	28920
15	terrigecera rgagrigada accadargge ecergagagg caradeadec acacroring	28980
	ctgtgtgete aegititiet taaagegiet gielggiitg eigelageat eaggeagaer	29040
	rgcagcagac tacatatgct cagccctgaa gtccttctag ggtgcatgtc tcttcagaar	29100
	ticagaaagt catcigigge tecaggaceg ceigeacter eceicigeeg egaggigea	29160
20	gacteragge tggggtggaa geaacgetta cetetgggae aagtataaca tgreggerte	29220
20	totttocoto tgtggotoca acctggacat aaaatagatg caagetgtgt aataaatatt	29280
	tcctcccgtc cacttagttc tcaacaataa ctactctgag agcacttatt aataggtggc	29340
	ttagacataa getttggete attececcae tagetettae ttetttaaet ettteaaace	29400
	attotgtgto ttocacatgg ttagttacct otcottocat cotggttego ttottoctto	29460
25	gagtegeeet cagtgtetet aggtgatget tgtaagatat tetttetaca aagetgagag	29520
	tggtggcact ctgggagttc aaagccagcc tgatctacac agcaagctcc aggatatcca	29580
	gggcaatgtt gggaaaacct ttctcaaaca aaaagagggg ttcagttgtc aggaggagac	29640
	ccatgggtta agaagtctag acgagccatg gtgatgcata cctttcatcc aagcacttag	29700
	gaggcaaaga aaggtgaaac tctttgactt tgaggccagc taggttacat agtgataccc	29760
30	tgcttagtgt gtgtgtgt gtgtgtgtgt gtgtgtgtgt gtgtgtaatt taaaagtcta	29820
	aaaatgcatt cttttaaaaa tatgtataag tatttgcctg cacatatgta tgtatgtatg	29880
	tataccatgt gtgtgtctgg tgctgaagga ctaggcatag actccctaga actagagtca	29940
	tagacaging tgacacicco caacccccca ccatgigggi gottgaagot aaactcctgi	30000
	cctttgtaaa gcagcaggtg tctatgaacc ctgaaccatc tctccagtct ccagatgtgc	30060
35	atteteaaag aggagteett catattteee taaaetgaae ateettatea gtgageatee	30120
	togagicaco adagotacig cadacoctot tagggaacat toactatica ottotacitg	30180
	gctcatgaaa cttaagtaca cacacacaaa cacacacaca cacacagagt catgcactca 3	30240

	caaaagcatg catgtacace attettatta gaetatgett tgetaaaaga ettteetaga	30300
	tactttaaaa catcacttot goottitggt gggcaggtto caagattggt actggcgtac	30300
	tggaaactga acaaggtaga gatctagaaa tcacagcagg tcagaagggc cagcctgtac	30360
	aagagagagt tecacacett ecaggaacae tgagcagggg getgggaeet tgeeteteag	30420
5	cccaagaaac tagtgcgttt cctgtatgca tgcctctcag agattccata agatctgcct	30480 30540
	totgocataa gatotootgo atooagacaa gootagggga agttgagagg otgootgagt	30540
	ctctcccaca ggccccttct tgcctggcag tattttttta tctggaggag aggaatcagg	30660
	gtgggaatga tcaaatacaa ttatcaagga aaaagtaaaa aacatatata tatatatt	30720
	aactgateta gggagetgge teageagtta agagttetgg etgeeettge tteagatett	30780
10	gotttgatto coagoaccoa catgatggot ttoaactgta tototgotto caggggatoo	30840
	aacageetet tetgaeetee atagaeaaga eetagteete tgeaagagea eeaaatgete	30900
	ttatetgttg atceatetet etageeteat gecagateat ttaaaaetae tggacaetgt	30960
	cccattttac gaagatgtca ctgcccagtc atttgccatg agtggatatt tcgattcttt	31020
	Ctaigitete accetigeaa titalaagaa agalatetge attigietee igagagaaca	31080
15	aagggtggag ggctactgag atggctctag gggtaaaggt gcttgccaca aaatctgaca	31140
	actiaagitt ggicitggaa tocacatggi ggagagagag aagagattoo ogtaagitgi	31200
	cotcaaactt cocacacatg tgotgtggot tatgtgtaac cocaataagt aaagatagtt	31260
	tradacacta cataaggtag ggtttcttca tgaccccaag gaatgatgcc cctgatagag	31320
241	cttatgctga aaccccatct ccattgtgcc atctggaaag agacaattgc atcccggaaa	31380
20	cagaatette atgaatggat taatgageta ttaagaaagt ggettggtta ttgcacatge	31440
	rggcggcgta atgacctcca ccatgatgtt atccagcatg aaggtcctca ccagaagtca	31500
	tacaaatett ettaggette cagagtegtg agcaaaaaaa gcacacetet aaataaatta	31560
	actagectea ggtagttaac cacegaaaat gaaccaagge agttetaata caaaaccaet	31620
25	tecettecet giteaaacea eagigeeeta tiaictaaaa galaaacite aageeaagei	31680
25	tttaggttgc cagtatttat gtaacaacaa ggcccgttga cacacatctg taactcctag	31740
	tactgggcct caggggcaga gacaggtgga gccctggagt ttgaattcca ggttctgtga	31800
	gaaactctgt ctgaaaagac aatatggtga gtgacccggg aggatatctg atattgactt	31860
	ctggccaaca cacagccatc tctgcacatc tgtagttgca agccttttgc actaagtttg 3	31920
30	gccagagtca gagtttgcaa gtgtttgtgg actgaatgca cgtgttgctg gtgatctaca 3	1980
30	aagtcaccct ccttctcaag ctagcagcac tggcttcggc cagctgctca ttcaagcctc 3	2040
	tttgcagagt catcacgggg atgggggagc agggcccctc cctagaacac caagcctgtg 3	2100
	gttgtttatt caggacatta ttgagggcca agatgacaga taactctatc acttggccaa 3	2160
	cagtegggtg ttgeggtgtt aggttattte tgtgtetgea gaaaacagtg caacetggae 3	2220
35	adaagaaata aatgatatca tttttcattc aggcaactag attccgtggt acaaaaggct 3	2280
33	ccctggggaa egaggccggg acagcgcggc tcctgagtcg ctatttccgt ctgtcaactt 3	2340
	ctctaatete ttgattteet ecctetgtet gttteettee tettgetggg geccagtgga 32	2400

	gtctgtgtac tcacagggag gagggtggca aagccctggt cototacggg ctgggggaag	32460
	gggggaaget gteggeeeag tgaettttte ecetttetet ttttettaga aaccagtete	32520
	aatttaagat aatgagtete eteatteaeg tgtgeteaet atteataggg aettateeae	32580
	ccccgccctg tcaatctggc taagtaagac aagtcaaatt taaaagggaa cgtttttcta	32640
5	aaaatgtggc tggaccgtgt gccggcacga aaccagggat ggcggtctaa gttacatgct	32700
	ctctgccagc cccggtgcct trtccrttcg gaaaggagac ccggaggtaa aacgaagttg	32760
	ccaacttitg atgatggtgt gcgccgggtg actctttaaa atgtcatgat as	32820
	agggaagget etteagggag teateragee erecetress and and a	32880
	tragitaget recaderggt coeffaced craftering coagrages as	32940
10	ggtttccgcc ctcatccacc ttgcccttrt agrtgctaga aagea	33000
	caggigggee ariggicaci cegetaceae igriaceaig gecaceaagg igreatitaa	33060
	atatgagete actgagteet gegggatgge tiggttggta atatgettge tgeaaaateg	33120
	tgagaactgg agttcaattc ccagcacatg gatgtatttc cagcacctgg aaggcagga 3	33180
	gragagatet taaageteet ggeragarag ceragertaa ttagtaatea graagagare 3	33240
15	Cigicicaag aaacaagaig gaacaicaaa ggicaaccic tigicicac acacacaaa;	33300
	acacacatgo acatacatoo acacacaggo aaacacatgo acacacotga acaccotoca 3	33360
	Caaatacata cataaaaaaa taaatacata cacacataca tacataca	33420
	cteettagte teetggetae getettgtea eccedactaa ggetteaact tettetatii 3	33480
20	cticatetig actecitigt actitigeatg certificag caaaggetif tetttaaare 3	3540
20	ECCGECATIC ATAMACTECE ECTAMATETE ELECCETGES CETETESTES ESCAPAGA 3	3600
	gataaagaca cacactacaa agtcaccgtg ggaccagttt attcacccac ccacccctgc 3	3660
	ticigiteat coggecaget aagtagteea aceteteigg tgetgtacee tggaccetgg 3	3720
	cricaccaca gerectecat geracecage cergeaaace treagectag cerergeric 3	3780
25	tocaaccage acaggeecag tetggettet atgteetaga aateteette atteteeca 3:	3840
25	Effected gaateracea cettettet ecetteteet gaeetetaat gtettggrea 3	3900
	aacgattaca aggaagccaa tgaaattagc agtttggggt acctcagagt cagcagggga 33	3960
	gorgggarga attoacattt coaggeottt gotttgotoo coggattorg acaggoagre 32	4020
	ccgaagctga gtccaggaag ctgaatttaa aatcacactc cagctgggtt ctgaggcagc 34	4080
30	cctaccacat cagctggccc tgactgagct gtgtctgggt ggcagtggtg ctggtggtgc 34	1140
30	tggtggtgct ggtggtggtg gtggtggtgg tggtggtggt ggtggt	1200
	ttttctgctt ttacaaaact tttctaattc ttatacaaag gacaaatctg cctcatatag 34	1260
	gcagaaagat gacttatgcc tatataagat ataaagatga ctttatgcca cttattagca 34	320
	atagitacig icaaaagtaa tictattiat acaccettat acatggtatt gettitgitg 34	380
25	gagactictaa aaticcagatt atgiatttaa aaaaaaatto oocagtoott aaaaggigaa 34	440
35	gaatggaccc agatagaagg tcacggcaca agtatggagt cggagtgtgg agtcctgcca 34	500
	atggtctgga cagaagcatc cagagagggt ccaagacaaa tgcctcgcct cctaaggaac 34	560

						atcagaccca	34520
						grgggggcc	34680
	aaggtgctgc	agtgggagcc	acatgagagg	tgatgtcttg	gagtcacctc	gggtaccatt	34740
	gtttagggag	gtggggattt	gtggtgtgga	gacaggcagc	ctcaaggatg	cttttcaaca	34800
5	atggttgatg	agttggaact	aaaacagggg	ccatcacact	ggctcccata	gctctgggct	34860
						tctgtgattc	34920
						gaccatcagg	34980
						taggatgctc	35040
	agctatctcc	tgagctggaa	ctattttagg	aataaggatt	atgecequee	ggggttggcc	35100
10	agcaccccag	cagcctgtgc	ttgcgtaaaa	gcaagtgctg	ttgatttatc	taaaaacaga	35160
	gccgtggacc	cacccacagg	acaagtatgt	atgcatctgt	ttcatqtatc	tgaaaagcga	35220
	cacaaccatt	tttcacatca	tggcatcttc	ctaaccccca	ttotttttta	tttrarres	35280
	ttgagacagg	gtttctctgt	gtagtcctgg	ctgtcctqqa	actcacttro	tagaccagge	35340
	tggcctcgaa	ctcagaaatc	ctgggattaa	aggtgtgtgc	caccacaccc	aggetagge	35400
15	cccattctta	atggtgatcc	agtggttgaa	atttcgggcc	acacacatot	CCattagge	
	ttagctgctg	tottotooo	* 200*	33320	acacacacge	ccactaggga	35460
		receegage	tacctggtac	aatctttatc	ccctggggcc	tgggctcctg	35520
	atccctgact	cgggcccgat	caagtccagt	tcctgggccc	gatcaagtcc	agttcctggg	35580
	cccgaacaag	tccagtccct	agctcgatta	gctcatcctg	gctccctggc	ctgttcttac	35640
	ttacactctt	ccccttgctc	tggacttgtt	gctttcttta	ctcaagttgt	ctgccacagt	35700
20	ccctaagcca	cctctgtaag	acaactaaga	taatacttcc	ctcaagcacg	gaaagtcctg	35760
	agtcaccaca	ccctctggag	gtgtgtggac	acatgttcat	gcgtgtggtt	gcgcttacgt	35820
	acgtgtgc					5 -	
	_						35828

<210> 18

25 <211> 9301

<212> DNA

<213> Homo sapien

<400> 18

- 30	t 2022000						
30	Lagaggagaa	gtctttgggg	agggtttgct	ctgagcacac	ccctttccct	ccctccgggg	60
	ctgagggaaa	catgggacca	gccctgcccc	agcctgtcct	cattggctgg	catgaagcag	120
						gctactggaa	180
	ggtggcgtgc	cctcctctgg	ctggtaccat	gcagctccca	ctggccctgt	gtctcgtctg	240
		cacacagcct					300
35	tgatgccacg	gaaatcatcc	ccgagctcgg	agagtacccc	gagcctccac	cggagctgga	360
		accatgaacc					420

	gaccaaaggt atggggtgga ggagagaatt cttagtaaaa gatcctgggg aggttttaga	450
	aacttetett tgggaggett ggaagaetgg ggtagaeeea gtgaagattg etggeetetg	480
	ccagcactgg tcgaggaaca gtcttgcctg gaggtggggg aagaatggct cgctggtgca	540
	gccttcaaat tcaggtgcag aggcatgagg caacagacgc tggtgagagc ccagggcagg	600 660
5	yaggacgetg gggtggtgag ggtatggcat cagggcatca gaacaggete aggggeteag	720
	aaaagaaaag gtttcaaaga atctcctcct gggaatatag gagccacgtc cagctgctgg	730
	taccactggg aagggaacaa ggtaagggag cotoccatoo acagaacago acctgtgggg	
	caceggacae tetatgetgg tggtggetgt ceceaecaea cagaeceaca tearggaare	840 900
	cccaggaggt gaacccccag ctcgaagggg aagaaacagg ttccaggaactt	960
10	. ggcagtgaga agagetgagg tgtgaaeetg gtttgateea aetgeaagat ageeetggtg	1020
	tyrgggggg tgtgggggac agateteeac aaageagtgg ggaggaagge cagagagge	1020
	cecergeagt gracetrace carageerae ceagagager ageacriqua agaaragaa	1140
	titteggeae agtittagee eetgacatgg gigeageiga gieeaggee iggaggggag	1200
	ayeageatee tetgtgeagg agtagggaea tetgteetea geageeacee cagteeaac	1260
15	cttgeeteat tecaggggag ggagaaggaa gaggaaceet gggtteetgg teaggeetge	1320
	acayagaago ccaggigaca gigigcaici ggototataa tiggoaggaa toolgaggas	1380
	arggggggt cigaaatgac acticagact aagagcticc cigiccicig gccattaicc	1440
	aygrygcaga gaagtccact gcccaggete ctggacccca gccctccccg cctcacaacc	1500
20	ryrryggadt atggggtgdt aaaaagggda actgdatggg aggddagdda ggadddtog	1560
20	rettedadat ggaggacaag ggegeeteee eecacagete eesttetagg caaggteage	1620
	ryggeredag egactgeetg aagggetgta aggaacecaa acacaaaatg tecacettge	1680
	rygactecca egagaggeca cageceetga ggaagecaca tgeteaaaac aaagteatga	1740
	telgeagagg aagtgeetgg cetaggggeg etattetega aaageegeaa aatgeegest	1800
25	recordage antigeococo tgaccacaca cacattecag ecetgeagag gtgaggatge	1860
23	addicageee acagaccaga aagcageeee agacgatgge agtggeeaca teterretee	1920
	rytyctiget etteagagig ggggiggggg giggeettet etgleecete tergettes	1980
	teledagaet attiticatt ettietigte acattggaae tateceeatg aaacettigg	2040
	aggregating gracticated gacgactage tattraaaaa geteecacee ateraagtee	2100
30	accalaggag acatggicaa ggigigigca ggggaicagg ccaggccicg gagcccaaic	2160
30	rectycologic cagggagtat caccatgagg cgcccattca gataacacag aacaagaaat	2220
	gradeceagea gagagecagg teaatgittg tggeagetga accigiaggi titgggicag	2280
	agercagge ecctarggra ggaaagraac gacagraaaa agcageeete agercearce	2340
	ceeageddag ceteccatgg atgetegaac geagageete caetettgee ggageeaaa	2400
35	garactggga ccccagggaa gtggagtccg gagatgcagc ccagcctttt gggcaagttc	2460
رر	treetergy craggeorea grattereat tgaraargag ggggtrggac acactgoort	2520
	tgattccttt caagtctaat gaattcctgt cctgatcacc tccccttcag tccctcgcct	2580

	ccacagcag	tgccctgatt	tattacctto	aattaacct	c tacteettt	c tocatoccct	2640
	gtccacccct	cccaagtggc	tggaaaagga	atttgggag	a agccagagc	c aggcagaagg	2700
	tgtgctgagt	acttaccctg	cccaggccag	ggaccctgc	g gcacaagtg	t ggcttaaatc	2760
	ataagaagac	cccagaagag	aaatgataat	aataataca	t aacagccga	c gctttcagct	2820
5	atatgtgcca	aatggtattt	tctgcattgc	gtgtgtaat	g gattaactc	g caatgottgg	2880
	ggcggcccat	tttgcagaca	ggaagaagag	agaggttaa	g gaacttgcc	aagatgacac	2940
	ctgcagtgag	cgatggagcc	ctggtgtttg	aaccccagc	a gtcatttgg	tccgaggga	3000
	cagggtgcgc	aggagagctt	tccaccagct	ctagagcat	tgggacctt	ctgcaataga	3060
	tgttcagggg	caaaagcctc	tggagacagg	cttggcaaaa	a gcagggctgg	ggtggagaga	3120
10	gacgggccgg	tccagggcag	gggtggccag	gcgggcggc	acceteaege	gegeetetet	3180
	ccacagacgt	gtccgagtac	agctgccgcg	agctgcactt	cacccgctac	gtgaccgatg	3240
	ggccgtgccg	cagcgccaag	ccggtcaccg	agctggtgtg	ctccggccag	rgeggeeegg	3300
	cgcgcctgct	gcccaacgcc	atcggccgcg	gcaagtggtg	gcgacctagt	gggcccgact	3360
	tccgctgcat	ccccgaccgc	taccgcgcgc	agcgcgtgca	gctgctgtgt	cccggtggtg	3420
15	aggcgccgcg	cgcgcgcaag	gtgcgcctgg	tggcctcgtg	caagtgcaag	cgcctcaccc	3480
	gcttccacaa	ccagtcggag	ctcaaggact	tcgggaccga	ggccgctcgg	ccgcagaagg	3540
	gccggaagcc	gcggccccgc	gcccggagcg	ccaaagccaa	ccaggccgag	ctggagaacg	3600
	cctactagag	cccgcccgcg	cccctcccca	ccggcgggcg	ccccggccct	gaacccgcgc	3660
20	cccacatttc	tgtcctctgc	gcgtggtttg	attgtttata	tttcattgta	aatgcctgca	3720
20	acccagggca	gggggctgag	accttccagg	ccctgaggaa	tcccgggcgc	cggcaaggcc	3780
	ccctcagcc	cgccagctga	ggggtcccac	ggggcagggg	agggaattga	gagtcacaga	3840
	cactgagcca	cgcagccccg	cctctggggc	cgcctacctt	tgctggtccc	acttcagagg	3900
	aggcagaaat	ggaagcattt	tcaccgccct	ggggtttaa	gggagcggtg	tgggagtggg	3960
25	aaagtccagg	gactggttaa	gaaagttgga	taagattccc	ccttgcacct	cgctgcccat	4020
23	cagaaagcct	gaggcgtgcc	cagagcacaa	gactgggggc	aactgtagat	gtggtttcta	4080
	greerggere	tgccactaac	ttgctgtgta	accttgaact	acacaattct	ccttcgggac	4140
	ctcaatttcc	actttgtaaa	atgagggtgg	aggtgggaat	aggatctcga	ggagactatt	4200
	ggcatatgat	tccaaggact	ccagtgcctt	ttgaatgggc	agaggtgaga	gagagaga	4260
30	gaaayagaga	gaatgaatgc	agttgcattg	attcagtgcc	aaggtcactt	ccagaattca	4320
50	gagetgtgat	gctctcttct	gacagccaaa	gatgaaaaac	aaacagaaaa	aaaaaagtaa	4380
	agagtctatt	tatggctgac	atatttacgg	ctgacaaact	cctggaagaa	gctatgctgc	4440
	Catcatons	ggcttccccg	gatgtttggc	tacctccacc	cctccatctc	aaagaaataa	4500
	at cooperate	tggggtagaa	aaggagaggg	tccgagggtg	gtgggaggga	tagaaatcac	4560
35	atccgcccca	acttcccaaa (gagcagcatc	cctcccccga	cccatagcca	tgttttaaag	4620
,,	tcaccttccg	aagagaagtg a	aaaggttcaa (ggacactggc	cttgcaggcc	cgagggagca	4680
	gccatcacaa	actcacagac (cagcacatcc (cttttgagac	accgccttct	gcccaccact	4740

	Cacagagag	a	• •				
	tracactas	a creetgeer	a gaaaacagc:	t tottactgo	t cttacatg:	g atggcatato	4800
	tacagagas	a agaacatta	t tgggggaaa:	a actacaagt	g ctgtacata	t gctgagaaac	4860
	tactttere	aatagetge	c acccaaaaat	ctttttgaa	a atcatttco	a gacaacctct	4920
5	tacteteegi	. gtagtttt:	a attgttaaaa	a aaaaaagt	t ttaaacaga	a gcacatgaca	4980
,	catgaaagc	tgcaggactg	g gtogttttt	tggcaattc	t tccacgtgg	g acttgtccac	5040
	aagaatgaaa	a gragrggttt	ttaaagagtt	aagttacat	a tttattttc	t cacttaagtt	5100
	atttatgcaa	a aagtttttct	tgtagagaat	gacaatgtt	a atattgctt	t atgaattaac	5160
	agtetgttet	tccagagtcc	agagacatto	g ttaataaag	a caatgaatc	a tgaccgaaag	5220
10	gatgtggtct	cattttgtca	accacacato	g acgtcattt	c tgtcaaagt	t gacacccttc	5280
10	tettggtead	tagageteca	accttggaca	cacctttgad	tgctctctg	g tagcccttat	5340
	ggcaattatg	tetteetttg	, aaaagtcatg	tttatccct	cctttccaa	a cccagaccgc	5400
	atttcttcac	ccagggcatg	gtaataacct	cagccttgta	a teetttage	agcotocot	5460
	ccatgctggc	ttccaaaatg	ctgttctcat	tgtatcacto	ccctgctcaa	aaqccttcca	5520
	tagetecese	ttgcccagga	tcaagtgcag	tttccctato	tgacatggga	ggccttctct	5580
15	gcttgactcc	cacctcccac	tccaccaagc	ttcctactga	ctccaaatgg	tcatgcagat	5640
	ccctgcttcc	ttagtttgcc	atccacactt	agcaccccca	ataactaato	: ctctttctt	5700
	aggattcaca	ttacttgtca	tatattaaca	taaccttcca	gagatgttcc	aatctcccat	5760
	gatecetete	tcctctgagg	ttccagccc	ttttgtctac	accactactt	tggttcctaa	5820
	ttctgttttc	catttgacag	tcattcatgg	aggaccagco	tggccaagtc	Ctqcttagta	5880
20	ctggcataga	caacacaaag	ccaagtacaa	ttcaggacca	gctcacagga	aacttcatct	5940
	tcttcgaagt	gtggatttga	tgcctcctgg	gtagaaatgt	aggatettea	aaagtgggcc	6000
	agcctcctgc	acttctctca	aagtctcgcc	tccccaaggt	gtcttaatag	tgctggatgc	6060
	tagctgagtt	agcatcttca	gatgaagagt	aaccctaaag	ttactcttca	gttgccctaa	6120
	ggtgggatgg	tcaactggaa	agctttaaat	taagtccagc	ctaccttggg	ggaacccacc	6180
25	cccacaaaga	aagctgaggt	ccctcctgat	gacttgtcag	tttaactacc	aataacccac	6240
	ttgaattaat	catcatcatc	aagtctttga	taggtgtgag	tgggtatcag	tggccggtcc	6300
	cttcctgggg	ctccagcccc	cgaggaggcc	tcagtgagcc	cctgcagaaa	atccargcar	6360
	catgagtgtc	tcagggccca	gaatatgaga	gcaggtagga	aacagagaca	tottocatoo	6420
	ctgagaggca	gtgcggtcca	gtgggtgggg	acacgggctc	tgggtcaggt	Frances	6480
30	tgtttgtttg	ttttgagaca	gagtctcgct	Ctattgccca	ggctggagra	Cagtgtcaca	
	atctcggctt	actgcaactt	ctgccttccc	ggattcaagt	gattctctt	Concaca	6540
	cagagtagct	gggattacag	gtgcgtgcca	CCacqcctaa	Ctaarrrra	tatttt	6600
	agagacgggg	tttcaccatg	ttggccaggc	tagtctcgaa	CtCttcacct	Canatana	6660
	gcctgcctcg	gcctcccaaa	gtgctgggat	tacaggcgra	agccaccaca	Caagegatet	6720
35	ggttggtgtt	tgaatctgag	gagactgaag	Caccaadaa	tragator++	tacasas	6780
	catacttggg	ctcagttcct	tgccctaccc	Ctcactroac	CEGGEE	Lycccacage	6840
			J		ccycctagaa	cctggtgggc	6900

	acatgggca	a taaccaggt	acactgttt	t graccaagt	g ttatgggaa	t ccaagatagg	6960
	agtaatttg	cictgtggagg	g ggatgaggg:	a tagiggita	gʻggaaagctt	c acaaagtggg	7020
	tgttgcttag	g agattttcca	a ggtggagaa	g ggggettet	a ggcagaagg	c atageceaag	7080
	caaagactgo	aagtgcatg	g ctgctcatgg	gtagaagag	a atccaccat	t cctcaacatg	7140
5	taccgagtco	ttgccatgtg	caaggcaaca	tgggggtac	c aggaattcc	a agcaatgtcc	7200
	aaacctaggg	g toigoittoi	gggacctgaa	gatacagga	t ggatcagcc	c aggctgcaat	7260
	cccattacca	cgaggggaa	aaaaacctga	aggotaaat	t gtaggtcgg	g ttagaggtta	7320
	tttatggaaa	gttatattct	acctacatgo	ggtctataa	g cctggcgcc	a atcagaaaag	7380
	gaacaaacaa	cagacctago	tgggagggg	agcattttg	t tgtaggggg	ggggcacatg	7440
10	ttctgggggt	acagccagac	tcagggcttg	tattaatagi	t ctgagagtaa	a gacagacaga	7500
	gggatagaag	gaaataggto	cctttctctc	tatatatata	totototot	actotototo	7560
	tctctcacac	acacacacag	acacacacac	acgetetgta	ggggtctact	tatgctccaa	7620
	gtacaaatca	ggccacattt	acacaaggag	gtaaaggaaa	agaacgttgg	, aggaqccaca	7680
	ggaccccaaa	attccctgtt	ttccttgaat	caggcaggac	ttacgcagct	gggagggtgg	7740
15	agagcctgca	gaagccacct	gcgagtaagc	caagttcaga	gtcacagaca	ccaaaagcto	7800
	gtgccatgtc	ccacacccgc	ccacctccca	catgataatt	gacacagece	tgtgctccac	7860
	aacccggctc	ccagatcatt	gattatagct	ctggggcctg	caccgtcctt	CCtgccacat	7920
	ccccacccca	ttcttggaac	ctgccctctg	tcttctccct	tgtccaaggg	caggcaaggg	7980
20	ctcagctatt	gggcagcttt	gaccaacagc	tgaggctcct	tttgtggctg	gagatgcagg	8040
20	aggcagggga	atattcctct	tagtcaatgc	gaccatgtgc	ctggtttgcc	cagggtggtc	8100
	togittacac	ctgtaggcca	agogtaatta	ttaacagctc	ccacttctac	tctaaaaaat	8160
	gacccaatct	gggcagtaaa	ttatatggtg	cccatgctat	taagagctgc	aacttgctgg	8220
	gegrägtage	tcacacctgt	aatcccagta	ctttgggacg	tcaaggcggg	tggatcacct	8280
25	gaggtcacga	gttagagact	ggcctggcca	gcatggcaaa	accccatctt	tactaaaaat	8340
23	acaaaatta	gcaaggcatg	gtggcatgca	cctgtaatcc	caggtactcg	ggaggctgag	8400
	acaggagaat	ggcttgaacc	caggaggcag	aggttgcagt	gagccaagat	tgtgccactg	8460
	cccccagec	ctggcaacag	agcaagactt	catctcaaaa	gaaaaaggat	actgtcaatc	8520
	actgcaggaa	gaacccaggt	aatgaatgag	gagaagagag	gggctgagtc	accatagtgg	8580
30	cagcaccgac	tcctgcagga	aaggcgagac	actgggtcat	gggtactgaa	gggtgccctg	8640
30	aatgacgttc	tgctttagag	accgaacctg	agccctgaaa	gtgcatgcct	gttcatgggt	8700
	gagagactaa	attcatcatt	ccttggcagg	tactgaatcc	tttcttacgg	ctgccctcca	8760
	argcccaatt	tccctacaat	tgtctggggt	gcctaagctt	ctgcccacca	agagggccag	8820
	agctggcagc	gagcagctgc	aggtaggaga	gataggtacc	cataagggag	gtgggaaaga	8880
35	yagatggaag	gagaggggtg	cagagcacac	acctcccctg	cctgacaact	tcctgagggc	8940
J.,	-ggtcatgcc	agcagattta	aggcggaggc	aggggagatg	gggcgggaga	ggaagtgaaa	9000
	aaygagaggg	tggggatgga	gaggaagaga	gggtgatcat	tcattcattc	cattgctact	9060

	gactggatge cagetgtgag ccaggeacea cectagetet gggcatgtgg ttgtaatett	
	ggagcotcat ggagctcaca gggagtgotg gcaaggagat ggataatgga oggataacaa	9120
	ataaacattt agtacaatgt ccgggaatgg aaagttctcg aaagaaaaat aaagctggtg	9180
	agcatataga caqccctqaa qqqqqqqqqqqqqqqqqqqqqqqqqqq	9240
5	agcatataga cagocotgaa ggoggocagg coaggoattt otgaggaggt ggoatttgag	9300
		9301
	<210> 19	
	<211> 21	
	<212> DNA	
10	<213> Artificial Sequence	
	<220>	
	<223> Primer for PCR	
15		
15	<400> 19	
	ccggagctgg agaacaacaa g	21
	<210> 20	
	<211> 19	
20	<212> DNA	
	<213> Artificial Sequence	
	Actificial Sequence	
	<220>	
	<223> PRimer for PCR	
25		
	<400> 20	
	gcactggccg gagcacacc	
		19
	<210> 21	
30	<211> 23	
	<212> DNA	
	<213> Artificial Sequence	
	·	
	<220>	
35	<223> Primer for PCR	

	<400> 21	
	aggccaaccg cgagaagatg acc	23
		23
_	<210> 22	
5	<211> 21	
	<212> DNA	
	<213> Artificial Sequence	
	<220>	
10	<223> Primer for PCR	
	<400> 22	
	gaagtccagg gcgacgtagc a	21
15	<210> 23	
	<211> 25	
	<212> DNA	
	<213> Artificial Sequence	
20	<220>	
	<223> Primer for PCR	
	400	
	<400> 23	
25	aagcttggta ccatgcagct cccac	25
	<210> 24	
	<211> 50	
	<212> DNA	
24)	<213> Artificial Sequence	
30		
	<220>	
	<223> Primer for PCR	
	<400> 24	
35	aagettetae tigicategi egicetigia giegiaggeg tietecagei	50

```
<210> 25
             <211> 19
             <212> DNA
             <213> Artificial Sequence
  5
             <220>
             <223> Primer for PCR
             <400> 25
 10
      gcactggccg gagcacacc
                                                                                19
            <210> 26
            <211> 39
            <212> DNA
15
            <213> Artificial Sequence
            <220>
            <223> Primer for PCR
20
            <400> 26
      gtcgtcggat ccatggggtg gcaggcgttc aagaatgat
                                                                               39
            <210> 27
            <211> 57
25
            <212> DNA
            <213> Artificial Sequence
            <220>
            <223> Primer for PCR
30
            <400> 27
     gtcgtcaagc tictacttgt catcgtcctt gtagtcgtag gcgttctcca gctcggc
                                                                              57
           <210> 28
35
           <211> 29
           <212> DNA
```

	<213> Artificial Sequence	
	<220>	
	<223> Primer for PCR	
5		
	<400> 28	
	gacttggatc ccaggggtgg caggcgttc	29
	<210> 29	
10	<211> 29	
	<212> DNA	
	<213> Artificial Sequence	
	<220>	
15	<223> Primer for PCR	
	<400> 29	
	agcataagct tctagtaggc gttctccag	29
20	<210> 30	
	<211> 29	
	<212> DNA	
	<213> Artificial Sequence	
25	<220>	
	<223> Primer for PCR	
	<400> 30	
	gacttggatc cgaagggaaa aagaaaggg	
30	5 555 1 223445333	29
	<210> 31	•
	<211> 29	
	<212> DNA	
	<213> Artificial Sequence	
35		
	<220>	

	<223> Primer for PCR	
	<400> 31	
	agcataagct tttaatccaa atcgatgga	
5		29
	<210> 32	
	<211> 33	
	<212> DNA	
	<213> Artificial Sequence	
10		
	<220>	
	<223> Primer for PCR	
	<400> 32	
15	actacgaget eggeecease acceateaac aag	33
		23
	<210> 33	
	<211> 34	
	<212> DNA	
20	<213> Artificial Sequence	
	<220>	
	<223> Primer for PCR	
25	<400> 33	
	acttagaage tttcagteet cageceeete ttee	34
	<210> 34	
	<211> 66	
30	<212> DNA	
	<213> Artificial Sequence	
	<220>	
	<223> Primer for PCR	
35	 	
	<400> 34	

	aatotggato cataacttog tatagoatao attataogaa gitatoigoa ggattogagg	60
	gcccct	66
	•	
	<210> 35	
5	<211> 82	
	<212> DNA	
	<213> Artificial Sequence	
	<220>	
10	<223> Primer for PCR	
	<400> 35	
	aatctgaatt ccaccggtgt taattaaata acttcgtata atgtatgcta tacgaagtta	60
	tagatotaga gtoagottot ga	82
15		04
	<210> 36	
	<211> 62	
	<212> DNA	
	<213> Artificial Sequence	
20		
	<220>	
	<223> Primer for PCR	
	<400> 36	
25	atttaggtga cactatagaa ctcgagcagc tgaagcttaa ccacatggtg gctcacaacc	60
	at	62
		62
	<210> 37	
	<211> 54	
30	<212> DNA	
	<213> Artificial Sequence	
	<220>	
	<223> Primer for PCR	
35		
	<400> 37	

	aacgacggcc agtgaatccg taatcatggt catgctgcca ggtggaggag ggca	5
	<210> 38	
	<211> 31 <212> DNA	
5	<213> Artificial Sequence	
•	<220>	
	<223> Primer for PCR	
10	<400> 38	
	attaccaceg gigacaceeg ettectgaca g	31
	<210> 39	
	<211> 61	
15	<212> DNA	
	<213> Artificial Sequence	
	<220>	
20	<223> Primer for PCR	
20	<400> 39	
	attacttaat taaacatgge gegecatatg geeggeeeet aattgeggeg categttaat t	60 61
25	<210> 40	
	<211> 34	
	<212> DNA	
	<213> Artificial Sequence	
30	<220>	
	<223> Primer for PCR	
	<400> 40	
35	attacggccg gccgcaaagg aattcaagat ctga	34
رد	<210> 41	

<211> 34

<212> DNA

<213> Artificial Sequence

5 <220>

<223> Primer for PCR

<400> 41

attacggcgc gcccctcaca ggccgcaccc agct

Inter Shall Application No PCT/US 99/27990

A. CLAS	SIFICATION OF SUBJECT	MATTER			703 99727990
IPC 7	C12N15/12 C07K16/22 G01N33/53	C07K14/51 C1201/68	C07K14/495 C12N15/62	C12N15/63 A61K38/18	C12N5/10 A61P19/10
According	to International Patent Clas	A01K67/027			
B. FIELD	S SEARCHED	omeanon tire) or to both	national classification ar	nd IPC	
Minimum o	COZV	assification system tollow	red by classification sym	Dolsi	
110 /	CU/K				
Document	ation searched other than m	inimum documentation to	the extent that such do	curnents are included in	the fields searched
Electronic	data base consulted during	ne international search	name of data base and		
				whole placifical, search	(erms used)
C. DOCUM	ENTS CONSIDERED TO B				
Category '	Citation of document, writ	indication, where appro	priate, of the relevant pa	issages	Relevant to claim No.
				- 	riolovanii to Claim No.
X	BIRREN ET AL	: "Homo sa	oiens chromos	ome	1,2,
	i 17, Clone Hi	RPC905N1, com	olete sequenc	e"	27-30
	14 November	1997 (1997-1)	I-14) YP0021	33305	
. 0	HEIDELBEKG [)E	- 1.7, XI 0021	55565	
	Ac AC003098 the whole do	Cumont			
	che whole de				ĺ
X	HILLIER ET A 1997" EMBL SEQUENC 19 May 1997 HEIDELBERG D Ac AA393939 the whole do	E DATABASE, (1997-05-19), E	erck EST Proj XP002133386	ect	1,2, 27-30
		_	-/		
			,		
	er documents are listed in th		X	Patent family members a	are listed in annex.
	egories of cited documents:		"T" later	decument or the bank of a few	
00.13100	nt defining the general state ered to be of particular releva	Ince	O1 P	TIVITIA MATE AUD DOT ID COU	the international filing date iffict with the application but ple or theory underlying the
"E" earlier oo filing da	ocument but published on or	after the international	"149	HIOH	
"L" document which may throw doubts on process along the considered				IN DA COUSIDELEG UOVEL C	or cannot be considered to in the document is taken alone
0.14.1011	or other special reason (as	Specified)	"Y" docur	ment of particular relevan	ce; the claimed invention ve an inventive step when the
•			4000	ATTENDED TO COMPONE OF WITH A	ne or more other such docu- ng obvious to a person skilled
	nt published prior to the inter an the priority date claimed		******	e art. nont member of the same	
Date of the ac	ctual completion of the intern	national search		of mailing of the internati	
	March 2000			07/04/2000	
Name and ma	alling address of the ISA European Patent Office.	0.0.5010.00	Autho	orized officer	
	NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040.				1
	Fax: (+31-70) 340-3016	С. СС. сроти,		Ceder, O	

Inte. onal Application No PCT/US 99/27990

Category ·	CHANGE OF GOLDENS WAS ASSESSED TO BE RELEVANT	
	Citation of document, with indication, where appropriate of the relevant passages	Relevant to claim No
K	BONALDO ET AL.: "Normalization and substraction: two approches to faciliate gene discovery" EMBL SEQUENCE DATABASE, 4 September 1998 (1998-09-04), XP002133484 HEIDELBERG DE AC AI113131 the whole document & BONALDO ET AL.: "Normalization and substraction: two approches to faciliate gene discovery" GENOME RES, vol. 6, no. 9, 1996, pages 791-806,	1,27-30
	US 5 780 263 A (ADAMS MARK D ET AL) 14 July 1998 (1998-07-14) cited in the application column 1, line 11 - line 13 column 1, line 40 - line 42 column 1, line 66 -column 2, line 47 column 9, line 50 - line 53 column 11, line 15 - line 37	1-22,32, 61-67, 73-79
	US 5 453 492 A (BUETZOW RALF ET AL) 26 September 1995 (1995-09-26) abstract column 3, line 60 -column 8, line 30	1-3,8,9, 11-13, 15-22, 59,61-67
	WO 91 13152 A (LUDWIG INST CANCER RES) 5 September 1991 (1991-09-05) the whole document	1-3,8, 11,13, 15,17,32
	HSU D R ET AL: "The Xenopus dorsalizing factor Gremlin identifies a novel family of secretes proteins that antagonize BMP activities" MOLECULAR CELL,US,CELL PRESS, CAMBRIDGE, MA, vol. 1, no. 5, April 1998 (1998-04), pages 673-683, XP002113640 ISSN: 1097-2765 cited in the application abstract page 676, left-hand column, line 10 - line 14	17
	WO 92 06693 A (FOX CHASE CANCER CENTER) 30 April 1992 (1992-04-30)	

International application No.

PCT/US 99/27990

Box	Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)
This Inte	ernational Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:
1. X	Claims Nos.: 57 and 58 because they relate to subject matter not required to be searched by this Authority, namely: see PCT/ISA/210
2.	Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3.	Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).
Box II	Observations where unity of invention is lacking (Continuation of item 2 of first sheet)
This Inte	ernational Searching Authority found multiple inventions in this international application, as follows:
1.	As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2.	As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.	As only some of the required additional search fees were timely paid by the applicant, this international Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4.	No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Remark	on Protest The additional search fees were accompanied by the applicant's protest. No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1998)

International Application No. PCT/US 99 27990

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 57 and 58 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.1

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

information on patent family members

Inte ional Application No PCT/US 99/27990

				101/0.	3 99/2/990
Patent document cited in search repor	t	Publication date		Patent family member(s)	Publication date
US 5780263	A 	14-07-1998	CA WO AU EP JP	2220912 A 9639486 A 2766595 A 0871705 A 11506918 T	12-12-1996 12-12-1996 24-12-1996 21-10-1996 22-06-1999
US 5453492	A	26-09-1995	NONE		
WO 9113152	Α	05-09-1991	US AU CA DE DE EP JP	5177197 A 649026 B 7449591 A 2076979 A 69131572 D 69131572 T 0517779 A 5504888 T	05-01-1993 12-05-1994 18-09-1991 28-08-1991 07-10-1999 23-12-1999 16-12-1992 29-07-1993
WO 9206693	A	30-04-1992	AU AU CA EP JP	662304 B 8957591 A 2094608 A 0554376 A 6502311 T	31-08-1995 20-05-1992 23-04-1992 11-08-1993 17-03-1994